Manipulating the mosquito microbiota to study its function

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Running head: Manipulating the mosquito microbiota

Summary/Abstract

Aedes aegypti mosquitoes are the main vectors of several arboviruses and are commonly used as models in mosquito biology and vector competence studies. The mosquito microbiota has an impact on different aspects of host physiology, including development, immunity and fecundity, in turn influencing the capability of the mosquito to transmit diseases. The composition of the microbiota is relatively simple in field mosquitoes and many of its bacterial members are culturable in the laboratory. Being able to manipulate the composition of the mosquito microbiota is essential to effectively investigate its effect on host physiology and vector competence. This protocol describes how to obtain gnotobiotic mosquitoes, *i.e.* mosquitoes with a known microbiota composition, and how to monitor the effect of a manipulated microbiota on mosquito development.

Key Words

Mosquito, microbiota, *Aedes aegypti*, development, gnotobiology, wing length

1. Introduction

The mosquito *Aedes aegypti* is the main vector of several arboviruses, including yellow fever, chikungunya, Zika and dengue viruses. The distribution of this mosquito species throughout all tropical and sub-tropical regions of the world and the ease of rearing it in the laboratory makes it a commonly-used model for mosquito biology and vector competence studies.

The mosquito microbiota is known to have a significant impact on several factors determining disease transmission, including mosquito vector competence (*i.e.* capability to acquire and transmit pathogens) and different aspects of mosquito physiology. The larval microbiota has a critical effect on host physiology, since larval development is only possible in the presence of a living microbiota [1]. The rescue of development in axenic (*i.e.* germ-free) larvae can be achieved with specific diet supplementations, but with significant delay in larval growth [2]. Exposure to different bacteria during larval development leads to significant differences in developmental rate, adult body size and egg production [3, 4]. The adult microbiota composition also influences host physiological traits such as lifespan, mating behavior and vector competence [5, 6].

The mosquito microbiota has a relatively low bacterial diversity, with four bacterial taxa representing on average 90% of the microbial population in the gut of a single mosquito [7]. Both culture-dependent and -independent analyses showed that the mosquito microbiota is mainly composed of Gram-negative bacteria, of which many have been cultured [7, 8, 9, 10]. Due to its culturability and simple composition, the mosquito microbiota can be manipulated in the laboratory and this represents a useful tool to study the role of the microbiota on mosquito physiology.

Here we describe a method to obtain gnotobiotic *Aedes aegypti* mosquitoes, *i.e.* mosquitoes that are colonized by a microbiota of known composition, in the present case composed of a single bacterial strain. In conventional rearing conditions, microbes are transmitted horizontally, between individuals sharing the same environment, or vertically, mostly via contamination of the surface of mosquito eggs by a female during egg laying. The egg cytoplasm is generally microbiologically sterile. When larvae hatch, they acquire their microbiota through ingestion of microorganisms present in the rearing water and/or on the egg envelope. Using this gnotobiology protocol, axenic *Aedes aegypti* larvae are obtained by surface sterilization of mosquito eggs (Figure 1). After addition of a bacterial culture to the rearing water, gnotobiotic larvae and mosquitoes are obtained and can be used to study the impact of a manipulated microbiota on mosquito development. Two different rearing methods are used depending on the parameters that need to be measured. Rearing larvae in a 24-well plate allows the follow-up of single individuals throughout development, while batch rearing in cell culture flasks is more time-efficient and therefore preferable when the measured parameters allow grouping. The experimental timeline of the protocol is indicated in Table 1.

Besides the rearing methodology, we provide an overview of different practices that are commonly used to estimate larval and mosquito development: larval length, duration of developmental stages, development success rate, adult sex ratio, adult wing length, and mosquito lifespan. Larval length is a parameter that is strictly dependent on larval rearing conditions. Notably, it varies significantly depending on larval density [11], rearing temperature [12] and diet [13]. In addition to larval length, larval development can also be quantified in terms of duration of developmental stages and of developmental success rate, which both depend on nutrition and microbiota [3, 14]. The sex ratio of emerged adults is also influenced by larval rearing conditions [11]. In *Aedes aegypti*, sex is completely genetically determined [15] and males have a faster larval development than females. Therefore, although the reasons behind why rearing conditions affect sex ratio have not been determined, one can theorize that the presence of a larval slow killer could result in a higher proportion of adult males. Wing length is commonly used as a read-out of adult body size, which is dependent on larval life-history traits. The adult body size is an important parameter, as it is known to directly correlate with blood meal volume and fecundity [16] and has been reported to be inversely correlated with dengue virus dissemination in the mosquito [17]. Finally, mosquito lifespan is a good indicator of the effect of the microbiota on the adult physiology. Considering that the transmitted pathogens develop for one to two weeks in the mosquito before being infectious for humans, mosquito lifespan is an important determinant of transmission.

Depending on the microbe and mosquito species used to generate gnotobiotic mosquitoes, this protocol should be adapted for the chosen host-microbe couple. In particular, culture media and microbial concentrations may vary from one microorganism to another and parameters of egg sterilization may vary between mosquito species. Here, we describe the materials and the procedures that we use to work with *Escherichia coli* in *Ae. aegypti*. However, several microbial strains have been used to produce gnotobiotic larvae of *Ae. aegypti* and *Anopheles gambiae* in independent laboratories [1, 3, 14].

2. Materials

Live organisms:

1. Glycerol *E. coli* stock kept at -80°C.
2. *Ae. aegypti* eggs, kept on a dry paper for 4 days to 3 months (*see* **Note 1**).

Media/solutions:

1. Liquid Luria-Bertani (LB) medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl*.
2. Solid LB medium: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 1.5 g/L agar*.
3. Sterile deionized water*.
4. 70% ethanol, prepared with sterile deionized water.
5. 1% bleach, freshly prepared from a tablet into sterile deionized water (bleach concentration refers to the active chlorine).
6. Sterile 5% (w/v) fish food (TetraMin Baby) in deionized water (*see* **Note 2**)*.
7. 4% paraformaldehyde in autoclaved phosphate-buffered saline*: PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4. Add paraformaldehyde just before use (manipulate concentrated paraformaldehyde under a fume hood).
8. Sterile 10% (w/v) sucrose solution*.

* autoclaved at 121°C for 30 minutes

Disposable materials:

1. Disposable vacuum filtration unit (the material and the pore size of the filtering membrane are not important, we generally use nitrocellulose 0.2 µm membranes).
2. Sterile 25 mL cell culture flasks with vented caps.
3. Sterile petri dishes.
4. Sterile 24-well plates.
5. Sterile plastic pipettes.
6. Sterile 15 mL tubes.
7. Microscope slides.
8. Coverslips.
9. Autoclavable polypropylene boxes used for plant culture, equipped with a larger tube (typically used for urine collection) for adult emergence and a smaller tube with a cotton roll for mosquito sugar feeding. Cover the filter on the top of the lid with gauze to avoid mosquito contamination. See Figure 2 for more details.

Other materials:

1. Microbiological safety cabinet (MSC).
2. Pump for vacuum filtration.
3. Dissection forceps.
4. Dissection microscope with camera.

3. Methods

3.1 The day before egg sterilization (**day 0**), revive the *E. coli* strain from the glycerol stock by streaking it onto an LB agar plate. Incubate the plate overnight at 37 °C.

3.2 **Sterilization of *Ae. aegypti* eggs – day 1** (this procedure should be conducted inside a MSC)

1. Transfer *Ae. aegypti* eggs onto the filter of a vacuum filtration apparatus system. Since the sterilization process can reduce the percentage of hatched eggs, we suggest exceeding the amount of eggs with respect to the required number of larvae by 10-15%. Note that the filtration unit does not need to be sterile as it is only used to remove sterilizing solutions. The same filtration unit can be used several times, as long as the filter is not visibly damaged.
2. From this point, proceed with egg sterilization without stopping between steps to avoid egg desiccation.
3. Add ~50 mL of 70% ethanol and incubate 5 minutes. During this incubation, mix the solution with a P1000 pipette tip to ensure that all eggs are well submerged in ethanol (*see* **Note 3**). Apply the vacuum to remove the ethanol.
4. Repeat the previous step with ~50 mL of 1% bleach (instead of ethanol) for 5 minutes, and then again with ~50 mL of 70% ethanol for another 5 minutes. The volumes of sterilizing solutions are approximate: the critical step is to ensure that the entirety of the egg surface is in contact with ethanol or bleach for the correct amount of time to ensure bactericidal activity.
5. Rinse eggs three times with abundant sterile deionized water, applying the vacuum each time to remove the water. This step is essential to remove any traces of sterilizing solutions.
6. Add sterile deionized water to the eggs and transfer them to a cell culture flask for larval emergence using a sterile plastic pipette. Keep the hatching larvae in their flask without any food until step 3.4.
7. To check the efficiency of egg surface sterilization, transfer 10-20 eggs to a sterile 15 mL conical tube containing 3 mL of LB and incubate shaking at 37 °C overnight.

3.3 Inoculate a single *E. coli* colony into LB and incubate shaking at 37°C overnight (for ~16 h, *see* **Notes 4 and 5**) – **day 1**

3.4 **Generation of gnotobiotic *Ae. aegypti* larvae – day 2**

1. Centrifuge the *E. coli* culture for 30 minutes at 3200 x g. Resuspend the pellet in the same volume of sterile deionized water. Dilute this suspension 1:5 and check that the resulting suspension contains approximately 10^8 CFU/mL by plating on LB-agar. This bacterial suspension will be added to larvae (*see* **Note 4**).
2. *24-well plate set-up*: transfer a single axenic larva to each well using a sterile plastic pipette. Add 2 mL of the bacterial suspension and 50 µL of the sterile diet suspension to each well.
3. *Flask set-up*: transfer 10 to 15 axenic larvae to each flask using a sterile plastic pipette. Add 15 mL of the bacterial suspension and 750 µL of the sterile diet suspension.
4. Keep an aliquot of axenic larvae in sterile water in a flask for a few days to ensure that larvae were not contaminated during manipulation. Axenic larvae are not able to grow

without bacteria: these larvae will molt to the second instar (whether food was added or not) only if contamination has occurred during the plate/flask set-up.

5. Ensure that plates and/or flasks are closed to maintain sterility. Place larvae in a climatic chamber (30°C with LD 12:12 h cycle) or in an insectary compartment specifically dedicated to microbiota-manipulated mosquitoes.

3.5 Maintaining gnotobiotic *Ae. aegypti* mosquitoes – days 8-10

When the pupal stage is reached, transfer pupae to the autoclaved polypropylene boxes (Figure 2). Pupae are placed in the larger tube, while a sterile 10% sucrose solution is added to the small tube containing the sterile cotton roll.

3.6 Monitoring the effect of the manipulated microbiota on mosquito development and survival

1. As a control, use eggs from the same batch that were not subjected to the sterilization process and hatched in non-sterile water. Alternatively, use gnotobiotic eggs from the same batch and from the same sterilization process that are colonized with a control microbe. The rearing conditions of non-sterile larvae should be as similar as possible to those used for the gnotobiotic rearing, using the same flasks or plates and the same sterilized diet. However, non-sterile controls should not be handled in microbiologically sterile conditions, *i.e.* on the bench rather than inside a MSC.

2. *Larval length*: 5 days post bacterial inoculation (**day 7**), larvae should have reached the fourth instar and body length can be used as a proxy for larval development (*see* **Note 6**). For this analysis, larvae can be reared either on 24-well plates or on flasks. Fix larvae for 20 minutes at room temperature in PBS with 4% paraformaldehyde. Using a clean plastic pipette, place the desired number of larvae on a microscope slide and remove as much liquid as possible. Several larvae can be placed on the same slide, using clean forceps. Take a picture rapidly to avoid larval desiccation (see **Note 7**). The larval length is considered the distance between the anterior border of the head and the posterior border of the last abdominal segment, excluding the siphon (Figure 3A).

3. *Duration of developmental stages*: larvae placed in single wells of a 24-well plate are inspected daily, three times per day. For each larva, the moment at which they molt is noted. In this way the duration of each larval instar and of the pupal stage can be determined for single individuals. A good indicator of larval molting is the presence and the size of exuviae in the well, therefore the larval stage can be deduced by counting the number of exuviae (1 exuvia = second instar, 2 exuviae = third instar, 3 exuviae = fourth instar). Since the first exuvia is often difficult to see, second instar larvae can also be distinguished from first instar larvae by their darker head.

4. *Wing length*: Collect adult mosquitoes 2 days post emergence, as younger mosquitoes may not have completely spread or dried wings, while older mosquitoes might start to have damaged wings. Both rearing conditions, whether in flasks or in plates, are relevant for this analysis. For convenience, adult mosquitoes can be kept at -20°C until analysis. The measurement can be conducted on both wings or on one specific wing. It is however important to discriminate between male and female mosquitoes because of the significant difference in their wing sizes (Figure 3B and 3C). Using dissection forceps, collect wings from several individuals and place them on a microscope slide. Use a coverslip to flatten samples and take a picture using the microscope camera. Wing length is defined as the distance between the alular notch and the radius 3 vein (Figure 3B and 3C).

5. *Percentage of developed larvae and sex ratio*: larvae reared in 24-well plates are left in the plates until the adult stage. For each individual, mark the sex of the adult mosquito or if larvae/pupae are undeveloped or dead. In our hands, gnotobiotic larvae generally reach

the adult stage at day 8-10 post bacterial inoculation. Since *Ae. aegypti* larvae might survive several days without developing, we set the end-point of the experiment at day 15 post bacterial inoculation, i.e. at least 5 days after observing the last pupation. This analysis allows operators to determine if the gnotobiotic rearing impacts the overall development of mosquitoes (*see* **Note 8**).

6. *Survival*: transfer pupae in autoclaved polypropylene boxes and provide sugar. Do not exceed 30 pupae per box. Inspect daily the boxes to count the number of dead and possibly determine their sex.

4. Notes

1. We use the New Orleans strain, but other *Ae. aegypti* strains have been used to produce gnotobiotic mosquitoes [3, 4].
2. We use TetraMin Baby but we do not exclude that other types of diet can be used. Since TetraMin Baby is insoluble, after autoclaving the diet solution we prepare 5 mL aliquots that are easier to resuspend. This provides a standardized amount of diet to larvae.
3. The incubation time with ethanol and bleach should not exceed 5 minutes: a longer treatment will result in a decrease of the percentage of hatched eggs.
4. The volumes of the bacterial culture and of the diluted bacterial suspension should be adapted to the number of gnotobiotic larvae required and to the type of larval rearing: consider that 50 mL of bacterial suspension are needed for each 24-well plate (24 larvae), while 15 mL are sufficient for each flask (10 to 15 larvae). For *E. coli* we use a 5-fold diluted bacterial suspension, therefore we inoculate bacteria in a LB volume corresponding to 1:5 of the final bacterial suspension volume (*e.g.* 200 mL of LB for 1 L of diluted bacterial suspension). Use a pre-inoculum if the bacterial culture volume is larger than 50 mL. Bacterial concentrations should be adjusted for each type of bacterium as the minimal amount to rescue larval development varies between microorganisms [14].
5. Ideally bacteria should not be in the death phase when they are given to larvae. For *E. coli* we decided to inoculate the bacterial culture 16 h before adding bacteria to sterile larvae. This time schedule should be adjusted for each type of bacterium.
6. Other morphometric parameters can be measured to estimate larval development. For example, the head capsule width or the ratio between thorax width and head capsule width are commonly used [18, 19].
7. We use a dissecting microscope equipped with a HD color camera (Euromex). After acquiring the image, we use the Image Focus software (Euromex) to measure larval length. As an alternative, the open source software ImageJ can be used to measure larval/wing lengths.
8. To determine if a real effect on sex ratio is present, you may determine the sex of undeveloped larvae and pupae. This can be achieved by PCR using the primers for the male-determining *Nix* gene described in [15].

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Figure captions

Figure 1. Experimental set-up to obtain gnotobiotic mosquitoes. Eggs are surface sterilized with three subsequent 5-minute washes in 70% ethanol, 1% bleach and 70% ethanol and rinsed in sterile water. A bacterial culture is added to axenic larvae to obtain gnotobiotic larvae and, subsequently, gnotobiotic adult mosquitoes. Gnotobiotic larvae can be reared in 24-well plates, if parameters on single individuals have to be monitored, or in cell culture flasks.

Figure 2. Preparation of sterile boxes for gnotobiotic adult mosquitoes. (**A**) Material needed to set-up the boxes: autoclavable polypropylene box for plant culture, larger autoclavable tube (typically used for urine collection), smaller autoclavable tube, cotton roll, gauze, filter paper cut to fit the bottom of the box. (**B**) The gauze is taped to cover the filter on the internal part of the lid to avoid mosquitoes being exposed to potential contaminants via their proboscis. (**C**) The filter paper is fixed to the bottom of the box with adhesive tape to collect mosquito excreta. The two tubes are also taped to the box sides: the larger one (red arrow) will allocate pupae, while in the smaller one (blue arrow) a cotton roll is placed for sugar feeding. (**D**) The prepared box is ready to be autoclaved: close the lid for approximately 3/4 of the surface, leaving one border of the lid opened. Close the lid immediately after sterilization. Alternatively, boxes can be autoclaved in autoclavable bags. After autoclave, let boxes dry inside a MSC. Before use, add the sterile 10% sucrose solution to the smaller tube with the cotton roll.

Figure 3. Measuring larval and wing lengths as a proxy for development. (**A**) Measurement of larval length of 5-day old *Ae. aegypti* gnotobiotic larvae. Larval length is measured from the anterior border of the head to the posterior border of the last abdominal segment, excluding the siphon. (**B, C**) Measurement of wing length on female (A) and male (B) *Ae. aegypti* gnotobiotic mosquitoes. Wing length is defined as the distance between the alular notch and the radius 3 vein. Bars: 1 mm.

Table caption

Table 1. Experimental timeline of the gnotobiology protocol.

Table 1

<table>
<tr><th>Day</th><th colspan="3">Protocol step</th></tr>
<tr><td>0</td><td colspan="3">Revive the bacterial strain (3.1)</td></tr>
<tr><td>1</td><td colspan="3">Sterilize Ae. aegypti eggs (3.2) and inoculate the bacterium (3.3)</td></tr>
<tr><td rowspan="2">2</td><td colspan="3">Generate gnotobiotic larvae (3.4)</td></tr>
<tr><td colspan="2">24-well plates</td><td>Culture flasks</td></tr>
<tr><td>3</td><td></td><td rowspan="3">Determine duration of developmental stages (3.6.3)</td><td></td></tr>
<tr><td>4</td><td></td><td></td></tr>
<tr><td>5</td><td></td><td></td></tr>
</table>

6			
7	Measure larval length (**3.6.2**)		Measure larval length (**3.6.2**)
8	Determine developmental success / sex ratio (**3.6.5**)		Transfer pupae to sterile boxes (**3.5**)
9			
10			
11	Start survival experiments (**3.6.6**)		
12			
13	Collect individuals for wing length determination (**3.6.4**)		