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Bacterial oral feeding assay with antibiotic-treated mosquitoes

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Corresponding Author:	Pa Wu
Corresponding Author's Institution:	
Corresponding Author E-Mail:	wupa@hunnu.edu.cn
Order of Authors:	Xinyi Liu
	Si Wu
	Wenqian Li
	Meihong Zhang
	Yan Wu
	Ning Zhou
	Pa Wu
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TITLE:

A bacterial oral feeding assay with antibiotic-treated mosquitoes

AUTHORS AND AFFILIATIONS:

Xinyi Liu[1,2], Si Wu[1,2], Wenqian Li[1,2], Meihong Zhang[1,2], Yan Wu[1,2], Ning Zhou[3], Pa Wu[1,2]

[1]Hunan Provincial Key Laboratory of Animal Intestinal Function and Regulation, College of Life Science, Hunan Normal University, Changsha, China

[2]State Key Laboratory of Developmental Biology of Freshwater Fish, Hunan Provincial Key Laboratory for Microbial Molecular Biology, College of Life Science, Hunan Normal University, Changsha, China

[3]Department of Infectious Diseases, the Second Xiangya Hospital, Central South University, Changsha, China

Corresponding Author:

Pa Wu

wupa@hunnu.edu.cn

KEYWORDS:

mosquito, midgut, microbiota, gut bacteria, antibiotic, sugar feeding, blood feeding, *Aedes aegypti*

SUMMARY:

This article presents a protocol to investigate the effect of individual mosquito gut bacteria, including isolation and identification of mosquito midgut cultivable microbes, antibiotic depletion of mosquito gut bacteria, and reintroduce one specific bacteria species.

ABSTRACT:

The mosquito midgut harbors a highly dynamic microbiome that affects the host metabolism, reproduction, fitness, and vector competence. Studies have been conducted to investigate the effect of gut microbes as a whole; however, different microbes could exert distinct effects toward the host. This article provides the methodology to study the effect of each specific mosquito gut microbe and the potential mechanism.

This protocol contains two parts. The first part introduces how to dissect the mosquito midgut, isolate cultivable bacteria colonies, and identify bacteria species. The second part provides the procedure to generate antibiotic-treated mosquitoes and reintroduce one specific bacteria species.

INTRODUCTION:

Mosquitoes are considered to be the most important vectors of human pathogenic diseases, transmitting over a hundred pathogens including Zika virus, Dengue virus, and *Plasmodium* parasites[1]. When mosquitoes take a blood meal to acquire nutrients for oviposition, they can accidentally ingest pathogens from an infected host via the digestive tract[2]. Importantly, the

mosquito midgut, which plays a pivotal role in both blood meal digestion and pathogen entrance, harbors a highly dynamic microbiome[3].

Several studies have characterized lab-reared and field-collected mosquito microbiota using either a culture-dependent method or a bacteria sequencing assay[4-6]. Species including *Pantoea*, *Serratia*, *Klebsiella*, *Elizabethkingia*, and *Enterococcus* are commonly isolated from mosquitoes in various studies[5,7-9]. Interestingly, mosquito gut microbiota fluctuates dynamically in both the community diversity and the amount of bacteria species, affected by the development stage, species, geographical origin, and feeding behavior[4]. Studies show that blood feeding dramatically increases the total bacterial load with rapid expansion of species from *Enterobacteriaceae* and a reduction in overall diversity[10,11]. In addition, mosquito gut microbiota of the larval stage is usually eradicated when the insect undergoes metamorphosis during pupation and eclosion; thus, newly emerged adult mosquitoes need to repopulate their microbiota[4].

Gut microbiota modulates insect physiology in various aspects, including nutrient absorption, immunity, development, reproduction, and vector competence[12]. Axenic mosquito larvae fail to develop beyond the first instar while a bacteria oral supply rescues development, indicating that the mosquito gut microbe is essential for larval development[13,14]. Besides, depletion of gut bacteria retards blood meal digestion and nutrient absorption, affects oocyte maturation, and decreases oviposition[15]. In addition, mosquitoes with gut microflora elicit higher immune responses compared to antibiotic-treated mosquitoes, with constantly elevated antimicrobial peptide expression against other pathogens to infect[16]. Antibiotics are usually orally administered to remove pan gut bacteria in these studies, and then experiments are conducted to compare the difference between axenic mosquitoes and mosquitoes with commensal microbes. However, the mosquito midgut harbors a diverse community of microbes, and each bacteria species could exert a distinct effect toward the host physiology.

Mosquito microbiota regulates vector competence with divergent effects. Colonization by *Proteus* isolated from field-derived mosquitoes of dengue-endemic areas confers upregulated antimicrobial peptide expression and resistance against dengue virus infection[16]. The entomopathogenic fungus *Beauveria bassiana* activates the Toll and JAK-STAT immune pathway against arbovirus infection[17]. By contrast, the fungus *Talaromyces* isolated from *Aedes aegypti* midgut facilitates dengue virus infection by modulating gut trypsin activity[18]. In addition, *Serratia marcescens* promotes arbovirus transmission through a secretory protein called *Sm*Enhancin, which digests the mucin layer on the intestinal epithelium of mosquitoes[19].

This procedure provides a systematic and intuitive method for dissection of the mosquito midgut, isolation of cultivable bacteria colonies, identification of the bacteria species, and reintroduction via oral feeding. It provides representative results of blood feeding with a commensal bacterium, *Chryseobacterium meningosepticum*, on mosquito ovary development and oviposition.

PROTOCOL:

1. Midgut dissection and cultivable bacteria isolation

1.1. Prepare the mosquito for dissection.

1.1.1. Collect the mosquitoes 7–9 days after emergence with an aspirator. Anesthetize the collected mosquitoes by subjecting them to a temperature of 4 °C for 3–5 min and keep the mosquitoes anesthetized in an ice-cold Petri dish until dissection.

1.2. Sterilize laboratory instruments and the mosquito surface.

1.2.1. Sterilize the experiment bench, dissecting microscope, forceps, and glass slide by spraying 75% ethanol to avoid contamination by bacteria from the environment.

1.2.2. Prepare a sterile Petri dish containing 75% ethanol and two Petri dishes containing sterile 1x phosphate buffered saline (1x PBS).

1.2.3. Surface-sterilize the mosquito by dipping and shaking them in 75% ethanol for 3 min, and rinse twice with 1x PBS buffer in case the ethanol affects the dissected gut flora.

1.3. Dissect the mosquito midgut.

1.3.1. Transfer the surface-sterilized mosquito onto the glass slide with a drop of sterile 1x PBS buffer. Dissect under the dissecting microscope.

1.3.2. Under the lower magnification of the dissecting microscope, carefully remove the legs, wings, and head of the mosquito to prevent escape. Remove the last somite of the mosquito by cutting directly, rather than pulling it, to prevent the digestive tract from breaking.

1.3.3. Clamp the mosquito's thorax and the end of the abdomen with forceps. Gently pull out the mosquito's digestive tract. The acquired digestive tract usually contains the crop, foregut, midgut, hindgut, and the Malpighian tubes.

1.3.4. Adjust the dissecting microscope to a higher magnification. Remove the crop, foregut, hindgut, and the Malpighian tubes from the dissected digestive tract to get the midgut of the mosquito.

1.3.5. Take care while removing the crop, as the crop inflates and resembles the midgut after the mosquito takes a sugar meal. The Malpighian tubes, a group of long and thin excretory tubes, are located at the boundary between the midgut and the hindgut.

1.4. Isolate gut bacteria.

1.4.1. Transfer the dissected midgut to a sterile 1.5 mL tube with 200 µL of sterile 1x PBS buffer.

1.4.2. Grind the midgut on a sterile bench thoroughly with a sterile pestle to allow complete releasing of gut bacteria to the buffer.

1.4.3. Serial dilute the homogenate to three 10-fold dilutions. Add 50 µL of each dilution to the LB (Luria broth) agar plate, and then spread it on the plate. If the midgut contains a high concentration of gut flora, it is essential to make more serial dilutions to pick out single colonies.

1.4.4. Incubate the plate at 37 °C for 1–2 days until single colonies are visible.

1.5. Species identification

1.5.1. Pick single colonies and inoculate them respectively into a 150 mL conical flask containing 50 mL of LB broth medium.

1.5.2. Shake the bacteria at 37 °C overnight.

1.5.3. Extract total DNA by bacterial genomic DNA extraction kit. Amplify 16S rDNA by polymerase chain reaction (PCR).

NOTE: There are multiple segments in 16S rDNA that are conserved. Based on these conserved regions, universal primers for bacteria can be designed to amplify 16S rDNA fragments of all bacteria. These primers are specific to bacteria, and the difference in the variable region of 16S rDNA can be used to distinguish different bacteria. Therefore, 16S rDNA is widely used for bacterial identification.

1.5.4. Recover and purify DNA fragments from PCR products by agarose gel electrophoresis and a commercial gel recovery kit.

1.5.5. Perform DNA sequencing on the purified DNA fragments to obtain bacterial gene sequences.

1.5.6. Compare the 16S rRNA gene sequence with the bacterial sequence available in GenBank. Select the Bacteria and Archaea database.

NOTE: Identification to the species level was defined as ≥99% 16S rDNA sequence similarity to the closest GenBank entry. The isolate was assigned to the corresponding genus when its 16S rDNA sequence similarity was <99% and ≥95%.

2. **Antibiotic treatment and bacterium reintroduction**

2.1. Prepare the antibiotic solution.

2.1.1. Weigh the required amount of sucrose, penicillin, and streptomycin to prepare a 10% sucrose solution including 20 units of penicillin and 20 μg of streptomycin per mL.

2.2. Feed mosquitoes with the antibiotic solution.

2.2.1. Anesthetize the mosquitoes in 4 °C for 3–5 min. Transfer the mosquitoes to a paper cup. Cover the top with gauze and enwind the gauze with tape to prevent mosquitoes from escaping.

2.2.2. Dip sterile cotton balls into the antibiotic solution and place them carefully on the mosquito cup. Squeeze the cotton balls before use to prevent mosquitoes from drowning in the dripping cotton balls.

2.2.3. Cover the cotton balls with a 10 cm Petri dish to prevent moisture evaporation. Replace the cotton balls twice a day for three consecutive days.

2.3. Confirm the effectiveness of the antibiotic treatment.

2.3.1. According to the above method, dissect the midgut of antibiotic-treated mosquitoes.

2.3.2. Transfer the dissected midguts to a sterile 1.5 mL tube with 200 μL of sterile 1x PBS buffer.

2.3.3. Grind midguts in a sterile bench thoroughly with a sterile pestle to allow complete release of gut bacteria to the buffer.

2.3.4. Extract the gut microbial DNA by bacterial genomic DNA extraction kit.

2.3.5. Perform bacterial quantitation by real-time quantitative PCR (qPCR) on genomic DNA using universal eubacteria primers (16S rRNA F: TCCTACGGGAGGCAGCAGT and R: GGACTACCAGGGTATCTAATCCTGTT).

2.4. Preparation of bacterial suspension

2.4.1. Measure the bacterial solution with a spectrophotometer. Add 1 OD (OD600) bacterial suspension into a 1 mL tube. Centrifuge the suspension at 5,000 x *g* for 5 min at 4 °C.

2.4.2. Discard the supernatant and add 1 mL of sterile 1x PBS buffer to 1.5 mL tube for suspension precipitation.

2.4.3. Centrifuge at 5,000 x *g* for 5 min at 4 °C; discard the supernatant.

2.4.4. Repeat steps 2.4.2 and 2.4.3.

2.4.5. Add 200 µL of sterile 1x PBS buffer to the bacterial precipitation.

2.4.6. Add 600 µL of 10% sucrose solution, 200 µL of 10 mm ATP (which acts as a phagostimulant), and 200 µL of bacterial suspension into a sterile 1.5 mL tube. Mix by vortexing. Alternatively, suspend bacterial pelleted in heat-inactivated blood.

2.4.7. Preparation of heat-inactivated blood

2.4.7.1. Collect fresh blood with an anticoagulant tube.

2.4.7.2. Take 2–3 mL of fresh blood. Centrifuge at 1,000 x *g* for 10 min at 4 °C to separate plasma and blood cells. Note that the blood will be lost during the centrifugation and washing process; there is a need to calculate the usage in advance.

2.4.7.3. Collect the plasma into a new 1.5 mL tube and heat-inactivate at 56 °C for 1 h.

2.4.7.4. Add 500 µL of sterile 1x PBS buffer to 1.5 mL tube for suspension of blood cells. Centrifuge at 1,000 x *g* for 10 min at 4 °C and then discard the supernatant.

2.4.7.5. Repeat step 2.4.7.4 twice.

2.4.7.6. Resuspend blood cells with heat-inactivated plasma to obtain heat-inactivated blood.

2.4.7.7. Follow steps 2.4.1 to 2.4.4 to get 1 OD bacterial pelleted.

2.4.7.8. Resuspend the bacteria pelleted with 1 mL of heat-inactivated blood.

2.5. Feed the mosquito with bacteria suspension.

2.5.1. Starve the mosquitoes for 24 h, allowing antibiotics to metabolize before feeding bacteria.

2.5.2. Assemble the membrane-feeding system.

2.5.3. Seal the sterilized feeder unit with a parafilm, alternatively a collagen membrane.

2.5.4. Put on a plastic ring and fix it with parafilm to avoid breaking due to friction during feeding.

2.5.5. Slowly add the prepared bacterial solution to the feeder unit. Note that only one well of the two-well feeder unit can be filled with the solution and cover the feeder unit only from the reagent dropping side.

2.5.6. Connect the feeder unit to a feeding system preheated to 37 °C, which simulates human temperature to attract mosquitoes. Place the feeding device on a paper cup filled with mosquitoes and feed for 90 min.

2.5.7. After feeding, anesthetize the mosquitoes by subjecting them to a temperature of 4 °C for 3–5 min and keep the mosquitoes anesthetized in an ice-cold Petri dish.

2.5.8. Pick the fully engorged mosquitoes for further studies.

REPRESENTATIVE RESULTS:

The midguts of mosquitoes treated with antibiotics and without antibiotics were taken out for DNA extraction, and qPCR was performed with universal bacterial primers. **Figure 1** shows the expression of bacterial 16S rRNA in the control group and antibiotic treatment group. The results show that about 98% of the gut bacteria have been removed, and the gut sterilization of penicillin and streptomycin was successful.

With the methods described, bacteria strains were isolated and identified. *C. meningosepticum* is a nonfermenting, oxidase-positive gram-negative aerobic bacillus, belonging to *Chryseobacterium*. **Figure 2** shows the average egg laid per mosquito after blood feeding of antibiotic-treated mosquitoes with *C. meningosepticum*. **Figure 3** shows the expression of ovarian development related genes 24 h after blood meal containing *C. meningosepticum*. An overview of the ovarian development related genes and primer sequences are listed in **Table 1**.

The strain isolated from intestinal bacteria from which intestinal bacteria was removed and then the fully engorged mosquitoes were divided into three groups. Five females in the first group and the second group were used to count the spawning amount, and 10 females in the third group were used to detect the gene expression related to ovarian development after 24 h of each female mosquito after feeding, respectively. The results show that there is no significant change in the egg production of the control group and the feeding group.

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FIGURE AND TABLE LEGENDS:

Figure 1: Expression of 16S rRNA after antibiotic treatment. The mosquitoes with untreated antibiotics served as the control group.

Figure 2: Effect of gut microbe on mosquito oviposition. The indicated bacteria strains were mixed with blood and fed to antibiotic-treated mosquitoes. The mosquitoes with untreated gut commensal microbes served as the control group. The data is presented as mean ± SEM.

Figure 3: Expression of reproduction related genes after blood feeding. Expression of Cathepsin B (**A**), Mitochondrial ATPase inhibitor (**B**), Ribosome biogenesis protein brix (**C**), and Serine/Threonine-protein kinase rio2 (**D**) after blood feeding of antibiotic-treated mosquitoes for 24 h with the indicated bacteria strains. The mosquitoes with untreated gut commensal microbe served as the control group. Each dot represents a mosquito and each line represents the median value of the group.

Table 1: Ovarian development related genes and primer sequences.

DISCUSSION:

Research on host-microbe interactions have found that different gut microbes affect their host physiology via divergent mechanisms. This article introduces the method to investigate the respective role of mosquito gut microbe, including dissecting mosquito midgut, culturing cultivable gut bacteria, antibiotic treatment, and reintroducing the bacteria of interest.

For successful antibiotic treatment, the following details must be considered in conducting the experiment. In this protocol, mosquitoes were treated using cotton balls moistened with a 10% sucrose solution including 20 units of penicillin and 20 µg of streptomycin per mL for 3 days[11,16]. Penicillin served as a broad-spectrum antibiotic against gram-positive bacteria, and streptomycin served as a broad-spectrum antibiotic against gram-negative bacteria[20]. Proper preservation of antibiotics is vital for efficient antibiotic treatment[21]. When stored at -18 °C, the stability of penicillin G is at least 3 m. Streptomycin is stable for at least 12 m when stored at 4 °C[22]. While differences in antibiotic brand or storage time may cause slight differences in microbial clearance, it is necessary to verify the efficiency after each antibiotic treatment. In addition to the qPCR, spreading the plate or using PCR are commonly used to evaluate the efficiency of antibiotic treatment[23,24]. Moreover, to prevent mosquitoes from being contaminated with other bacteria after antibiotic treatment, the feeding assay needs to be performed with sterilized equipment assembled under a super clean bench.

Before bacterial oral feeding, the mosquitoes should be starved for 24 h to allow the antibiotics to be metabolized[25]. This not only helps the mosquitoes ingest bacteria liquid, but also prevents the antibiotics from killing the bacteria.

Admittedly, this protocol is mainly for investigating the effect of cultivable bacteria. To study the intestinal microbiota of vertebrate and invertebrate host, reintroduction of cultivable bacteria to antibiotic-treated host is commonly used in recent researches[8,26,27]. In addition, the initial identification of cultured intestinal bacteria is generally based on the characteristics of colony size, shape, color, edge, opacity, height, and consistency[28]. For non-culturable bacteria, high throughput sequencing-based metagenomic approaches are likely to provide comprehensive information on the total composition of the midgut microbiota[29-31]. However, the interplay between non-culturable bacteria and the host remains largely unexplored.

This article offers two alternative options to study the effect of gut microbe on mosquito oviposition, mix the bacteria with heat-inactivated blood or implantation of the bacteria via

sugar feeding followed by blood feeding. The representative results in this manuscript adopted the first option, while the latter method generated similar results. Various studies could be conducted after oral feeding with one specific bacteria strain. Subsequent assay could be used to investigate how microbial factor modulates mosquito locomotor behavior. Protein quantification assay could be followed to study the effects of different bacteria on digestion. With minor modifications, this method could be used to study the respective effects of various microbes toward mosquito physiology, including nutrient absorption, immunity, development, reproduction, and vector competence.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

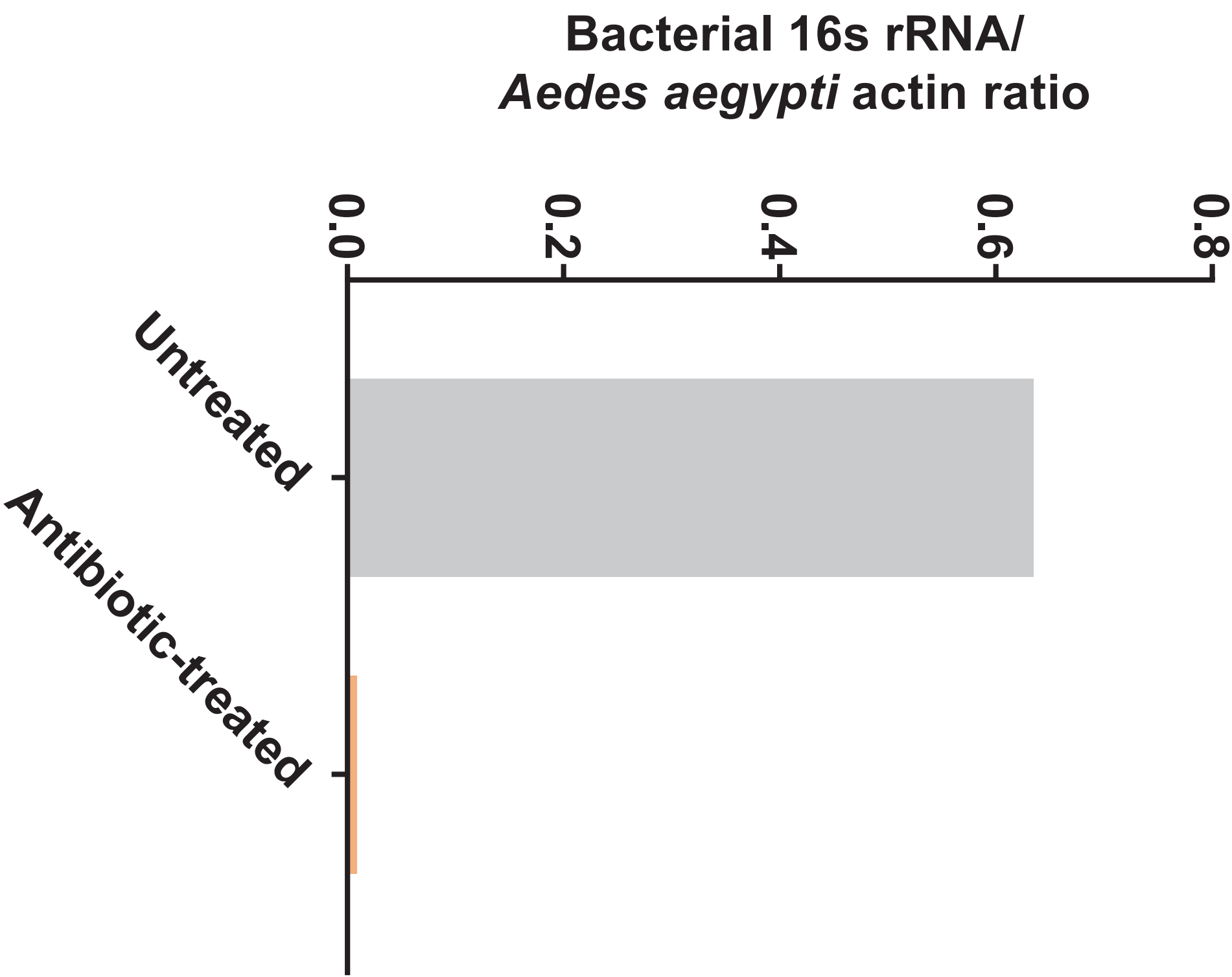

Figure 2

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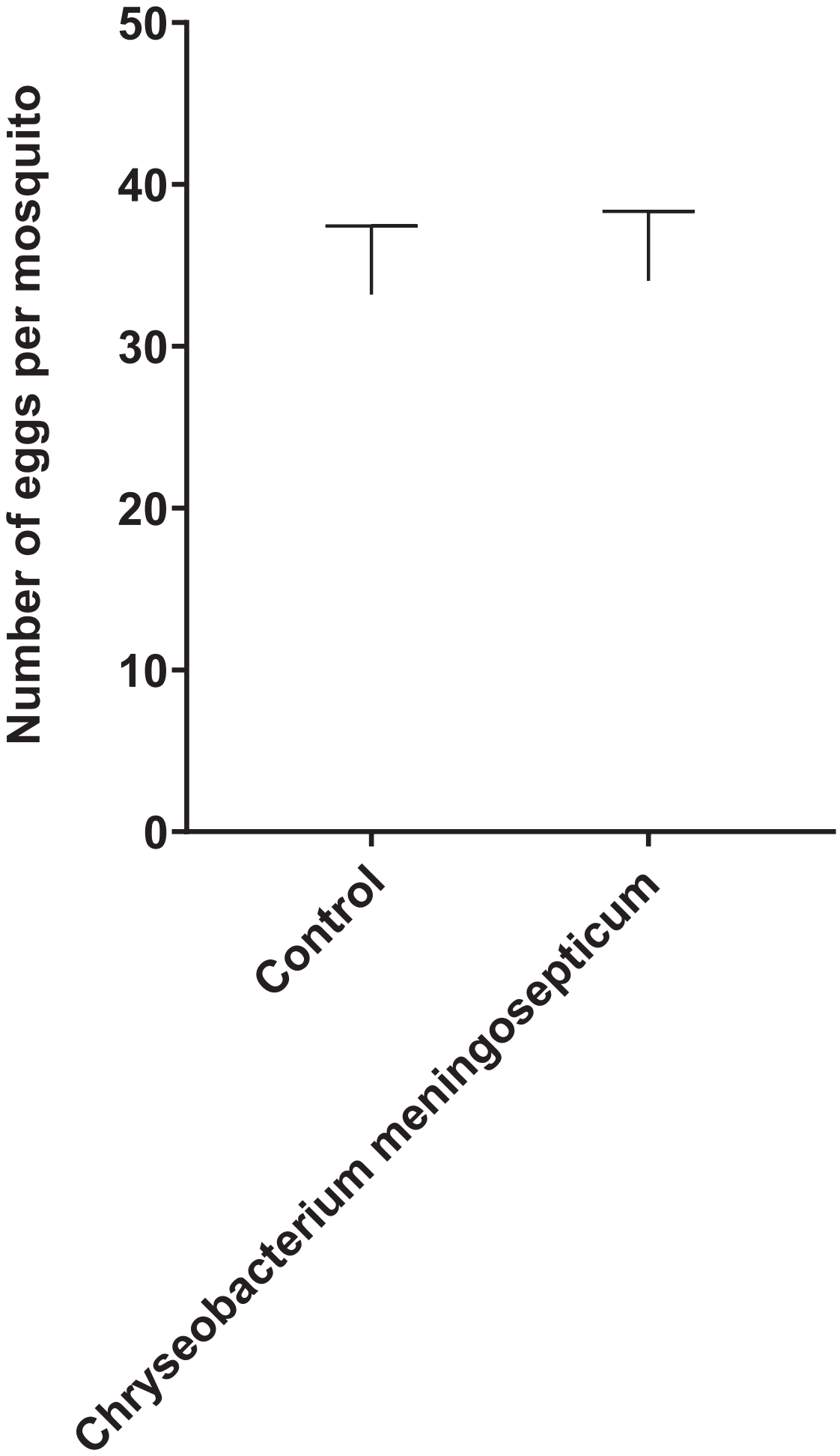

Figure 3

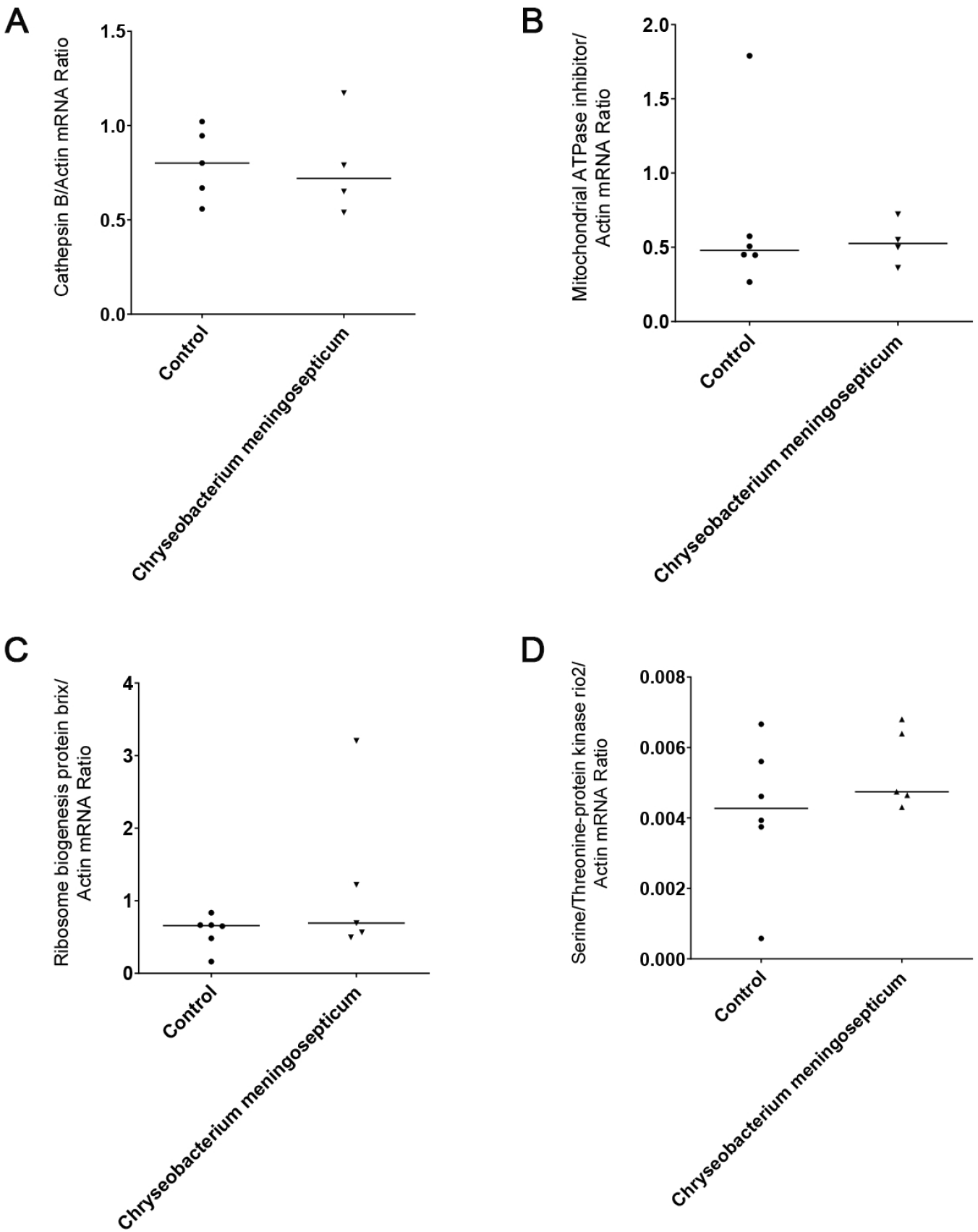

Table 1

Name	gene number
Cathepsin B	AAEL009642
Mitochondrial ATPase inhibitor	AAEL004284
Ribosome biogenesis protein brix	AAEL001917
Serine/Threonine-protein kinase	AAEL011114

primer sequence
Forward primer-GAGGGAAAGTTCGATGTGGA Reverse primer-AATCCCACATCCACCCAGTA
Forward primer-CAACTGCACAAGCTGAAGGA
Reverse primer-ACGTGCGATAGCTTCTTCGT
Forward primer-GAACAGCACAAGCGAATGAA
Reverse primer-TTGGCCTTGAGAGTCGTCTT
Forward primer-GAGGAGAAAGCAGCACAACC Reverse primer-TCGAATGGCTTTTCCATTTC

Name of Material/Equipment	Company
Adenosine 5’-triphosphate disodium salt hydrate	Sigma
Aedes aegypti	
Anticoagulant tube	BD Vacutainer
Centrifuge tube	Sangon Biotech
Cotton balls	
Disposable Tissue Grinding Pestle	Sangon Biotech
Ethanol absolute	Paini
Forceps	RWD

Hemotek Membrane Feeding System	Hemotek
Incubator shaker	
Inoculation Loops	Sangon Biotech
LB Agar Powder	Sangon Biotech
LB Broth Powder	Sangon Biotech
Microscope	Zeiss
Paper cup	
Parafilm	Sangon Biotech
Petri dish	Sangon Biotech

Penicillin G procaine salt hydrate	Sangon Biotech
Single Channal Pipettor	Gilson
Streptomycin sulfate	Sangon Biotech
Sucrose	Sangon Biotech
TIANamp Bacteria DNA Kit	TIANGEN
Utility Fabric-Mosquito Netting White	
Vortex mixer	Scintic Industries
1.5ml EP tube	Sangon Biotech
10X PBS buffer	Sangon Biotech

Catalog Number	Comments/Description
A2383	Adenosine 5’-triphosphate disodium salt hydrate has been used to prepare adenosine triphosphate (ATP) standard solutions
	Female mosquitoes
363095	Collect fresh blood
F601620-0010	1.5 ml, Natural, Graduated, Sterile
F619072-0001	70 mm Long, Conical, Blue, Sterile
	Dilute it to 75% ethanol
F11029	Dissection

	Components of the feeding system, including Hemotek temperature controller, feeder-housing assembly, metal feeder assembled.
ZQZY-78AN	
F619312-0001	10 µl, Yellow
A507003	Tryptone 10.0 g； Yeast Extract 5.0 g； NaCl 10.0 g； Agar 15.0 g.
A507002	Tryptone 10.0 g； Yeast Extract 5.0 g； NaCl 10.0 g.
Stemi508	
	Place mosquito
F104002	4 inx 125 ft
F611203	

A606248	White powder. Soluble in water, soluble in methanol, slightly soluble in water, ethanol
A610494	Streptomycin sulfate is a glucosamine antibiotic that interferes with the synthesis of prokaryotic proteins.
A502792	Soluble in water, ethanol and methanol, slightly soluble in glycerol and pyridine.
DP302	Extract DNA
S1-0246	
F600620	
E607016	This product is a 10X solution. Please dilute it 10 times before use. The pH value is 7.4.

Dear Editors,

Thank you for the suggestion on our manuscript (JoVE61341R2). We have revised the question about the title card in the video. The revised video entitled “Bacterial oral feeding assay with antibiotic-treated mosquitoes” is submitted for your consideration. Below is our detailed answer about the video.

Regarding the video:

#1. *• 00:00 Title card: The text around the affiliations gets bolder and more pixelated after one second. When it first comes up, it looks good. Can you check the title card and remove any extra effects or attributes that may be causing this unintended pixelation?*

#1 Answer: Thanks for the suggestion. We have checked the title card and corrected this effect. The opacity of all text on the title card has become 100%. The text of the title card is now clear.