CHAPTER II

MATERIALS AND METHODS

1. MATERIALS

1.1 For preparation of blood containing *B. malayi* microfilariae

Hank's Balanced Salt Solution (HBSS, pH 7.2-7.4)

1.2 For light and scanning electron microscopic studies

Phosphate buffer saline solution (PBS, pH 7.4), Giemsa's stain (pH 7.2), 2.5% glutaraldehyde in PBS (pH 7.4), 1% osmium tetroxide, 30%, 50%, 70%, 80%, 90%, 95% and absolute ethyl alcohol

2. METHODS

2.1 Mosquitoes

Ae. aegypti (Thailand strain) was used in this study. The mosquitoes were maintained at standard lighting conditions of 12 h light/dark, relative humidity of 70-80% and temperature of 27°C (±2°C). Adult mosquitoes were provided with a 10% (w/v) sucrose solution until they were 5-7 days old. Then, they were fasted for 12 h prior to feeding on anesthetized mice. After that, the mosquitoes were mated. The gravid female mosquitoes were encouraged to oviposit in a plastic cup of natural water with wet filter paper lining the inside (Figure 2.1a). Eggs were placed in a white plastic tray (25×35×6 cm) containing 1,500 ml of natural water and exposed to a 40 watt light (Figure 2.1b). After hatching, one hundred first instar larvae were fed on

dog food once a day (Figure 2.1c). The resulting pupae were transferred to a plastic cup containing natural water (Figure 2.1d) and placed in a cage for emergence (Figure 2.1e). The mosquitoes were transferred to another place by using sucker and a plastic cup (Figure 2.1f). These have been colonized continuously under laboratory conditions for several generations in the insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, since 2012. This mosquito strain was found to be refractory to nocturnally subperiodic *B. malayi* when previously tested. In the present study, this phenotype was confirmed before performing experiments. A group of female mosquitoes was infected as described below and dissected 14 days post-infected blood meal (PIBM) after feeding on *B. malayi*- infected blood and none were found infected.

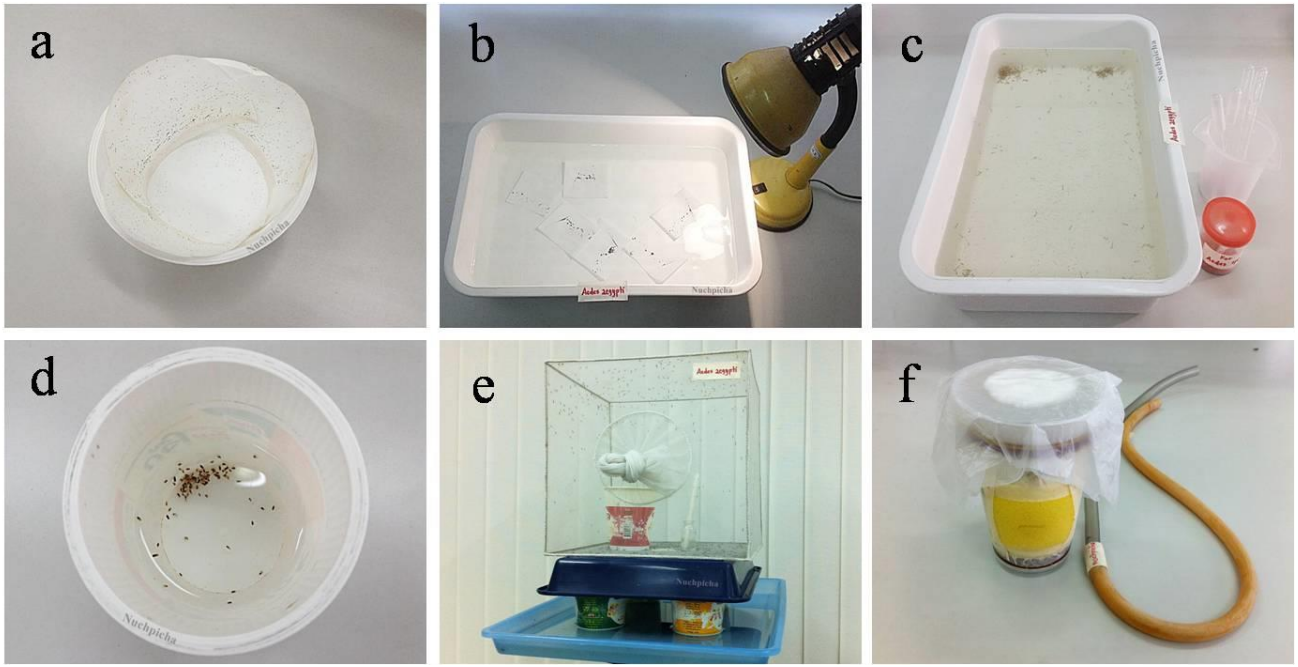

Figure 2.1 Equipment for mosquito rearing. (a) Plastic cup of natural water with wet filter paper lining the inside for gravid female mosquitoes to lay eggs. (b) Eggs placed in a white plastic tray containing 1,500 ml of natural water and exposed to a 40 watt light. (c) White plastic rearing tray containing natural water, transfer pipette and dog food. (d) Plastic container for holding pupae (e) Adult rearing cage and bottle with cotton wick containing 10% sucrose solution. (f) Plastic container and sucker.

2.2 Source of *Brugia malayi* microfilariae

The nocturnally subperiodic *B. malayi* parasite strain originated from a 20-year-old female resident of Bang Paw district, Narathiwat province, southern Thailand. From 1982 until 1986 the strain was maintained by experimental infection of domestic cats at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. Then the strain was transferred to Mongolian jirds (*Meriones unguiculatus*), and has since been maintained at the animal house of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (Choochote et al. 1986; Saeung and Choochote 2013).

2.3 Preparation of blood containing *B. malayi* microfilariae and infection of mosquitoes

The jirds were anesthetized deeply with ethylene ether and inoculated intraperitoneally with infective larvae of *B. malayi*. The microfilariae were collected after at least three months (Choochote et al. 1991) by injecting 3 ml of Hank's Balanced Salt Solution (HBSS, pH 7.2-7.4) into the peritoneal cavity before withdrawing by peritoneal washing. A 0.5 ml volume of peritoneal washings enriched with microfilariae was mixed with 10 ml of human heparinized blood (10 units of heparin/ml of blood), which had been taken from donors. The microfilarial density was then adjusted to approximately 200-300 microfilariae/20 µl in human-heparinized blood, which was used for artificial mosquito feeding. The reason for this adjustment was based on previous experiments that yielded satisfactory susceptibility of *Oc. togoi* to nocturnally subperiodic *B. malayi* (susceptibility rates of 70-95%, Jariyapan et al. 2013). This agreed with experiments reporting the susceptibility of *Anopheles sinensis* to periodic *B. malayi* using a microfilarial density of 5, 10, 20 and 50 microfilariae/20

µl, with susceptibility rates of 30%, 65%, 93%, and 100%, respectively (Luo and Qu 1990). Three day-old adult females of *Ae. aegypti* (fasted for 12 h) were infected by artificial feeding on blood-containing *B. malayi* microfilariae, using the techniques and apparatus described by Chomcharn et al. (1980).

2.4 Exsheathment studies and preparation of samples for light microscopy (LM)

To determine if exsheathment of *B. malayi* microfilariae occurred within the midgut, engorged mosquitoes were dissected and their midguts recovered in phosphate buffer saline solution (PBS) at different time points after the blood meal (5 min, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 12 h, 18 h, 24 h, 36 h, 48 h, 72 h, and 96 h), taking care not to damage the midgut. Ten excised intact midguts were processed for each time point. Each individual midgut was carefully transferred to a new glass slide and then opened. The ingested blood meal from the midgut was used to prepare a thick blood film, which was dried out, dehemoglobinized, fixed with absolute methanol, and stained with Giemsa's stain (pH 7.2). The remaining mosquito body and fluids were thoroughly teased apart in PBS, allowed to air dry, fixed with absolute methanol, and Giemsa-stained. The microfilariae in each sample were counted and examined for the presence or absence of their sheaths under a light microscope. Photographs of the microfilariae were taken using a digital camera (Cannon, Tokyo, Japan) attached to the light microscope. Triplicate experiments were performed and counts were rechecked by more than one observer.

2.5 Preparation of samples for scanning electron microscopy (SEM)

Engorged mosquitoes were dissected as described above and ten samples from each time point were processed for SEM. Dissected midguts were fixed overnight

with a solution of 2.5% glutaraldehyde in PBS, pH 7.4 at 4°C to accomplish primary fixation. The samples were then rinsed twice with PBS at 10 minute intervals and post-fixed for 2 h in a solution of 1% osmium tetroxide. Post-fixation was followed by rinsing twice with PBS. Water was replaced in the specimens with alcohol by subjecting them to alcoholic concentrations of 30%, 50%, 70%, 80%, 90%, and 95%. Then the specimens were placed in absolute alcohol for two 12 h periods. After that, specimens were placed in acetone for 2 h, subjected to critical point drying and attached to aluminum stubs with double-sided tape. Finally, they were coated with gold in a sputter-coating apparatus before being viewed with a scanning electron microscope (JEOL JSM-5910LV, JEOL Ltd., Japan). To observe the interface between the midgut surface and the microfilarial infected blood meal, some fixed samples were fractured before being coated with gold, while others were gently opened and their contents were washed out before fixation with PBS.