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Original article

Development of a simple and convenient feeding device to infect *Aedes aegypti* mosquitoes with *Brugia pahangi* microfilariae derived from the peritoneal cavity of Mongolian jirds

Yoshinori Mitsui[1*] and Mitsumasa Miura[1]

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Abstract: It has become difficult in recent years to conduct the direct feeding of mosquitoes on animals because of ethical considerations related to animal experimentation. Thus, the artificial feeding of mosquitoes on blood meals is an important technique in studies on the oral infection of mosquitoes to agents. Since Rutledge *et al.* (1964) devised the artificial membrane-feeding technique, several artificial membrane-feeding methods have been developed to increase the feeding rates of mosquitoes on blood meals. The purpose of the present study is to develop a simple and convenient device for the artificial feeding of mosquitoes. We designed a device using Kimwipe®, a coverglass, the lid of a plastic dish and a 50 ml Erlenmeyer flask. The efficacy was assessed by the infection rate of mosquitoes to *Brugia pahangi* microfilariae (MF) derived from the peritoneal cavity of Mongolian jirds. Immediately after the feeding of mosquitoes on MF by the new device, the MF infection rate of mosquitoes was 50 - 81%. On day 14 post-feeding, 51 - 94% of mosquitoes harbored third-stage infective larvae. The components needed to construct the device for artificial feeding of mosquitoes are generally available in laboratories. Furthermore, no elaborate modification of materials is necessary in making the feeding device. Therefore, this simple and convenient artificial feeding device promises to be applicable for experimental infection of mosquitoes not only with *B. pahangi* MF but also with other agents such as malaria and viruses.

INTRODUCTION

Mosquitoes are an important vector for the maintenance and production of parasites that are infective for vertebrate hosts. Mosquitoes reared in the laboratory require feeding on blood meals, but ethical considerations related to animal experimentation often preclude direct feeding of mosquitoes on animals. Thus, the artificial feeding of mosquitoes on blood meals is an important technique in studies on the oral infection of mosquitoes to agents. Since Rutledge *et al.* devised the artificial membrane-feeding technique [1], several artificial membrane-feeding methods have been developed [2 - 5]. To increase the feeding rate of mosquitoes on blood meals, several types of membrane have been implemented [1, 2, 6, 7], including parafilm®, which is widely used as an inexpensive and easily obtainable material [8 - 12]. However, warming tends to result in increased permeability and fragility of the parafilm® stretched over the feeding apparatus and to subsequent leakage of blood or medium in the feeding apparatus.

Microfilariae (MF) from the peritoneal cavity of Mongolian jirds infected with *Brugia pahangi* intraperitoneally provide a rich and convenient resource for experimental studies [13]. Chuang *et al.* reported that cavity-borne MF [14], ingested to mosquitoes by an artificial feeding apparatus, developed to third-stage infective larvae (L3). Subsequently, Schrater *et al.* confirmed that the MF ingested by mosquitoes developed to L3 [15], although the rate of cavity-borne MF penetrating the midgut walls of mosquitoes was lower than that of blood-borne MF. In addition, because the artificial feeding methods used by Chuang *et al.* and Schrater *et al.* were neither easy nor convenient [14, 15], cavity-borne MF have not been used routinely in laboratories to produce L3. To utilize the cavity-borne MF for mass production of L3 and to maintain the *B. pahangi* lifecycle, it is essential to develop an efficient artificial feeding method.

1. Department of Parasitology, Institute of Tropical Medicine, Nagasaki University
*Corresponding Author:
Department of Parasitology, Institute of Tropical Medicine, Nagasaki University, Sakamoto, Nagasaki, 852-8523 Japan.
E-mail: ymitsui@nagasaki-u.ac.jp
Tel: +81-95-819-7823
Fax : +81-95-819-7824

The objective of the present study is to develop a simple device for the artificial feeding of mosquitoes. The feeding efficacy of mosquitoes using the new device was assessed by determining the infection rates of mosquitoes to *B. pahangi* MF collected from the peritoneal cavity of Mongolian jirds and the infection rates of mosquitoes with L3.

MATERIALS AND METHODS

Chemicals and medium

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Sigma Chemical Co., (St. Louis, Missouri, USA) and Gibco-BRL Life Technologies, (Grand Island, New York, USA), respectively. Glucose and disodium adenosine 5-triphosphate (ATP) were obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). The artificial blood meal (ABM) used for mosquito feeding experiments was RPMI-1640 medium (pH: 7.4) supplemented with 2 mM ATP, 2% glucose and 10% FBS. All the other chemicals used were of reagent grade.

Parasite strain

The filarial parasite *B. pahangi* had been maintained in Mongolian jirds (*Meriones unguiculatus*) and *Aedes aegypti* (Liverpool strain) mosquitoes in the Institute of Tropical Medicine, Nagasaki University. Jirds were infected with 200 *B. pahangi* L3 by the intraperitoneal route. Three months later, the jirds were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg). MF were collected by rinsing the peritoneal cavity with 5 ml of RPMI-1640 medium. The rising was centrifuged at 1,000 rpm for 7 min. The precipitated MF were used for the experiments dealing with the artificial feeding of mosquitoes.

Mosquitoes

Ae. aegypti mosquitoes (Liverpool strain) maintained in the Institute of Tropical Medicine, Nagasaki University were used throughout this study. Adult mosquitoes were reared in cages (20 x 20 x 30 cm) covered with a screen mesh (39 mesh/10 mm, polyester fibers) at ambient temperature (26±1℃) with 85±10% relative humidity, and fed on fine solid sugar and water separately. Female adult mosquitoes aged 7 - 8 days were starved 24 h prior to the artificial feeding experiments.

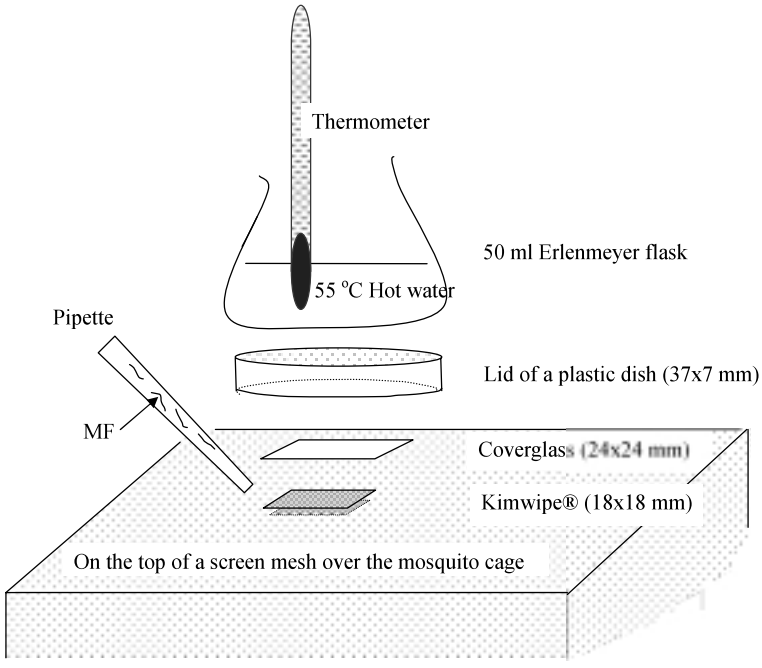

Fig. 1. Schematic diagram of the device for artificial feeding of mosquitoes using two pieces of Kimwipe®, a coverglass, the lid of a plastic dish and a 50 ml Erlenmeyer flask. The procedure is as follows:

1. Place two pieces of Kimwipe® (18 x18 mm) on top of a mosquito cage (39 mesh/10 mm).
2. Apply 300 µl, and an additional 200 - 500 µl of the artificial blood meal (ABM) containing microfilariae (MF) to the Kimwipe® with a pipette.
3. Cover the wet Kimwipe® with a coverglass (24 x 24 mm).
4. Cover the surface of the coverglass with the lid of a plastic dish (37 x 7 mm).
5. Warm the surface of the lid of a plastic dish with 55℃ hot water in a 50 ml Erlenmeyer flask.

Ingestion of Brugia pahangi microfilariae by Aedes aegypti mosquitoes through the Kimwipe®-coverglass feeding device

A schematic diagram of the feeding device and procedure is presented in Fig. 1. First of all, two pieces of Kimwipe® (Nippon Paper Crecia, Tokyo, Japan) paper cut to 18 x 18 mm were piled on the screen mesh of a mosquito rearing cage. Three hundred µl of the ABM containing MF (MF density: around 1,000 per 10 µl) was absorbed into the Kimwipe® papers, and a coverglass (24 x 24 mm, Matsunami, Tokyo, Japan) was attached to the wet papers. The coverglass was covered with the lid of a plastic dish (Sumilon Dish, Sumitomo Bakelite Co., Tokyo, Japan; 37 mm in diameter), onto the inner surface of which one human breath was expelled. The outer surface of the plastic dish was warmed with 55℃ hot water in a 50 ml Erlenmeyer flask. Mosquitoes were immediately attracted to the wet Kimwipe® papers from the inside of the mosquito rearing cage. During the feeding of mosquitoes for 2 - 3 h, an additional 200 - 500 µl of the ABM containing MF was applied to the Kimwipe® paper. After the feeding period, regardless of whether they had fed on the ABM or not, the mosquitoes were maintained with fine solid sugar and water separately at 26±1℃ for 14 days. Seven batches of 35 - 110 female mosquitoes were used for the artificial feeding trials.

The infectivity of Brugia pahangi microfilariae to Aedes aegypti mosquitoes and the development of the ingested microfilariae to third- stage infective larvae

Immediately after the feeding of mosquitoes on the ABM containing MF, around 10 mosquitoes were randomly sampled in each trial. Then, each sample was individually dissected in 50 µl of RPMI-1640 medium on a slide glass under a stereomicroscope to count the number of MF ingested by mosquitoes.

On day 14 post-feeding, all surviving mosquitoes in each trial were individually dissected in 200 µl of RPMI-1640 medium in the well of a 24-well plate (Sumilon, Sumitomo Bakelite Co., Tokyo, Japan) at room temperature. Then, the L3 were counted under a stereomicroscope.

The infection rate of mosquitoes with MF or L3 was expressed as a percentage of the number of mosquitoes infected with MF or L3 divided by the number of examined mosquitoes. The mean number of MF ingested or L3 harbored by mosquitoes was calculated as the number of MF or L3 in mosquitoes was divided by the number of examined mosquitoes.

RESULTS

Immediately after mosquitoes were exposed to MF using the Kimwipe® - coverglass feeding device for 2 - 3 h, 10 - 12 mosquitoes were randomly selected from each trial group. The mosquitoes were individually dissected in 50 µl of RPMI-1640 medium on slide glasses to count the *B. pahangi* MF ingested by each mosquito. The MF infection rate of the mosquitoes is shown in Table 1. In the 7 repeated trials, 50 - 82% of mosquitoes ingested MF. The mean number of MF ingested by a mosquito ranged from 12 to 198. During the 14-day period after the artificial feeding, 24 -

Table 1. The rate and intensity of infection of *Aedes aegypti* mosquitoes with *Brugia pahangi* microfilariae (MF) or third-stage infective larvae (L3) after the artificial feeding of mosquitoes using the developed device.

Trial	Number of mosquitoes in a cage	MF density per 10 µl of ABM*	Mosquitoes examined immediately after feeding		Mortality of mosquito for 14 days (%) ‡	Mosquitoes examined on day 14 post - feeding	
			Infection rate of mosquitoes with MF (%)**	Mean number of MF ingested by mosquito †		Infection rate of mosquitoes with L3 (%)**	Mean number of L3 harbored by mosquito †
1	92	970	7/10 (70.0)	11.8±4.7	25/82 (30.5)	29/57 (50.9)	2.8±0.8
2	98	1,070	6/10 (60.0)	24.1±19.7	31/88 (35.5)	33/57 (57.9)	2.7±0.6
3	105	724	8/10 (80.0)	22.0±7.2	30/95 (31.6)	54/65 (83.1)	5.4±0.7
4	66	1,036	6/12 (50.0)	27.6±11.4	20/54 (37.0)	28/34 (82.4)	2.8±0.5
5	35	883	5/10 (50.0)	47.5±21.7	9/25 (36.0)	15/16 (93.8)	2.5±0.5
6	110	996	9/11 (81.8)	198.3±59.3	64/99 (64.6)	33/35 (94.4)	9.1±1.3
7	48	1,072	6/10 (60.0)	54.8±20.8	9/38 (23.7)	27/29 (93.1)	3.4±0.6

* Total volume of ABM used for each feeding trial was 500 - 800 µl. ABM: RPMI-1640 medium supplemented by 2 mM ATP, 2% glucose and 10% FBS.
**The number of mosquitoes ingesting MF and harboring L3 are divided by the number of randomly sampled and all surviving mosquitoes, respectively.
† Total number of MF ingested and L3 harbored by mosquitoes are divided by the total number of examined mosquitoes. Data are expressed as mean ± standard error.
‡ The number of dead mosquitoes is divided by the number of mosquitoes remaining in a cage after being examined for MF ingestion.

65% of mosquitoes died. The infection rate and infection intensity of mosquitoes with L3 are shown in Table 1. On day 14 post-feeding, all surviving mosquitoes were dissected in 200 µl of RPMI-1640 medium to observe the existence of L3. The percentage of mosquitoes harboring L3 was 51 - 94. The mean number of L3 harbored by mosquitoes was 3 - 9.

DISCUSSION

The artificial-membrane feeding method using a Rutledge-type feeder and parafilm® is a popular method for the artificial feeding of mosquitoes [1]. However, warming the feeding apparatus to around 37℃ increases the permeability of the parafilm® attached to the apparatus and leads to subsequent leakage of blood or medium in the apparatus. By contrast, there was no leakage of ABM in the newly developed feeding system, although some evaporation and extension of the ABM was observed after 1 h feeding. The minimum volume of ABM used in the new feeding device was 500 µl, which was less than that (2.5 ml) in the Rutledge-type feeding system for one feeding trial [7].

Chuang *et al.* revealed that ingested MF developed to L3 in mosquitoes when the mosquitoes fed on only phosphate buffered saline (PBS) containing MF [14]. Since then, PBS has not been used as an ABM for artificial infection of mosquitoes to MF. Generally, fresh or defibrinated blood has been used as blood meals for the artificial feeding of mosquitoes on MF [16, 17]. In the present study, RPMI-1640 medium containing 2 mM ATP, 2% glucose and 10% FBS was used as the ABM in order to simplify and facilitate the artificial feeding method for the infection of mosquitoes with MF. FBS and glucose were added as nutrition for MF, and ATP was added as a phagostimulant to promote mosquito feeding [8]. As shown in Table 1, 50 - 82% of mosquitoes ingested MF in the ABM. The feeding response of *Ae. aegypti* mosquitoes on the ABM was higher than that (32%) in the blood-parafilm® feeding system reported by Failloux et al. [8]. The feeding rate of mosquitoes in the present study was similar to that (32 - 75%) of *Ae. aegypti* on blood through membranes of animal-derived skins [7]. The feeding efficacy of mosquitoes in the present method was higher than or similar to that of the membrane-feeding systems. Despite the similar density of MF used in each trial, the mean number of MF ingested by mosquitoes markedly varied, ranging from 12 to 198. In addition, 55℃ hot water was used as an attractant (Fig 1). Mosquitoes were more attracted to 55℃ than to 37℃ hot water.

In the present study, 65% of mosquitoes died in trial 6 during the 14-day period post-feeding, while 24 - 37% of mosquitoes died in the other trials (Table 1). The mean number of MF ingested by mosquitoes was 198 in trial 6 and 12 - 55 in the other trials. The high mortality rate of mosquitoes in trial 6 might be due to the higher number of MF ingested by the mosquitoes. This finding is consistent with the observation that mortality increased in mosquitoes heavily infected with MF [18 - 21].

The mean number of MF ingested by mosquitoes was 12 - 198, whereas the mean number of L3 recovered from mosquitoes after 14 days' artificial feeding was 3 - 9. Less than one fourth (5 - 25%) of the cavity-borne MF ingested by mosquitoes is estimated to develop to L3. On the other hand, Schrater *et al.* reported that 13 - 33% of the cavity-borne MF penetrated the midgut wall of mosquitoes [15]. The development rates in the present study were similar to or less than those observed in the study of Schrater *et al.* [15]. The lower development rates might be due to the lack of nutrients in the ABM used for feeding.

Finally, the infectivity of L3 produced by the new artificial methods was assessed. Three jirds were inoculated intraperitoneally with 200 L3 produced by the present artificial feeding system. Three months later, the adult worms and emerging MF were observed in all three jirds. This result suggests that the L3 produced by the new feeding method can be utilized to maintain the life-cycle of *B. pahangi* in laboratories.

In the present study, pieces of Kimwipe®, a coverglass, the lid of a plastic dish and a 50 ml Erlenmeyer flask (Fig. 1) were used to construct the simple device for artificial feeding of mosquitoes. These components are generally available in laboratories. Furthermore, no elaborate modification of materials is necessary for the feeding device. Therefore, this simple and convenient artificial feeding device promises to be applicable for experimental infection of mosquitoes not only with MF but also with other agents such as malaria and viruses.

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