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Development of a cheap and simple artificial feeding device for studying dengue virus transmission in *Aedes aegypti* mosquito at the resource-poor setups

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Abstract

Background & Objectives: Adult female *Aedes aegypti* mosquitoes require blood for development of their eggs to replenish their offspring. During blood feeding, ingestion of pathogens can occur which transmits the pathogens to the host during subsequent blood feeding. To study transmission and pathogen growth in mosquitoes, different feeding systems are utilized which includes live animals or feeding devices. These artificial systems are expensive and have several difficulties. In this study, a cheap and simplified artificial feeding device was developed to study pathogen transmission in the resource-poor setups and tested for its effectiveness.

Methods: An artificial feeding device comprises a “feeding container” and a “siphon” was developed using household and general laboratory materials. To test the effectiveness, the feeding device was examined and compared with a natural feeding method using pigeon. The transmissibility of dengue virus in *Aedes aegypti* was evaluated using the device after ingestion of infectious blood followed by detection of Dengue virus in mosquitoes using Real-time PCR.

Results & Conclusions: Feeding rate, fecundity and hatchability rates of *Aedes aegypti* using the feeding device observed in present study were same as the natural feeding method ($p > 0.05$). Dengue virus RNA could detect in 50% of *Aedes aegypti* mosquito pools. Besides the effective performance of the device, this newly developed device is made from house hold materials which made the device very inexpensive and user-friendly. It is anticipated that in resource-poor set up this device can be used with minor improvisations to study pathogen transmission in mosquitoes.

Keywords: *Aedes aegypti*, dengue virus, feeding device, feeding rate, fecundity, hatchability, transmissibility

Introduction

The mosquito-borne pathogens are acquired with a blood meal from the vertebrate host [1]. These organisms are subjects for different types of basic and applied research worldwide for development of innovative strategies for controlling disease transmission by mosquitos. These mosquitoes are reared in cages at laboratory facilities. These anautogenous insects require the blood meal for producing their eggs to complete the life cycle. For performing different experiments in laboratories, the mosquitoes are fed on various live animals such as guinea pig, mice, sheep, pigs, chickens, and pigeons [2-5]. The blood feeding success in mosquitoes is influenced by complete body emanations including body heat, odor, and moisture [6-7]. To maintain the correct temperature of the blood, some other artificial membrane devices; such as Hemotek membrane feeder [8] or glass feeder apparatus [9] were developed which contain blood surrounded by hot water supply. These artificial feeding systems are expensive, fragile and difficult to assemble for experiments [10]. These facilities are not affordable for all laboratory set ups because of limitations of funds, especially in the resource-poor setups [11].

The *Aedes aegypti* is an autogenous mosquito [12]. It is one of the main vectors of Dengue virus [13]. There are some experimental reports on transmitting pathogens including Dengue virus through artificial feeding device [14-16] or directly fed on skin of dengue infected patients [16].

These procedures using costly instruments or feeding on patients infected with the pathogen may not be workable in all research settings. Therefore, there is an urgent need to develop cheap and user-friendly artificial membrane blood feeding system for studying pathogen transmission of mosquitoes by using at the resource-poor setups.

In this study, a cheap, simplified artificial feeding device was developed for mosquito feeding and its effectiveness was investigated for three parameters of fitness, namely blood feeding rate (BFR), fecundity and hatchability of *Aedes aegypti* and compared with a natural (N) direct feeding method using pigeon. In addition, the transmissibility of Dengue virus in *Aedes aegypti* mosquito was investigated using the newly developed membrane feeding device by observing the presence of Dengue virus in *Aedes aegypti* mosquitoes after ingesting blood containing Dengue virus.

Materials & Methods

Ethics statement

All experiments with mosquitoes were conducted in an insectarium at the Department of Virology, Bangabandhu Sheikh Mujib Medical University (BSMMU), in compliance with the Bangladesh Animal Welfare Act 1920. For caring and handling of the used in the present study, the Guide for the Care and Use of Laboratory Animals (8th edition) published by the National Research Council, USA was followed [17]. Pigeons were purchased from a bird market, maintained in a cage and fed on a seed-based diet i.e. mixture of rice, wheat and maize and water. After mosquito feeding, the pigeons were freed in the nature. Written informed consent was taken from the adult Dengue patients whose plasma were used for the experiments. All the experiments were performed with the approval of an Institutional Review Board (IRB) of BSMMU, Dhaka, Bangladesh (IRB-BSMMU/2015/5310).

Structural component, assembly and feeding through the device

The newly developed feeding device comprises a “feeding container” and a “siphon” (Fig 1, 2). The container is a two-chambered plastic cylindrical container, which is used for the household purpose. Its upper bigger chamber termed as the heating chamber has the capacity of holding ~60 ml of hot water, while the smaller chamber below the heating chamber termed as blood chamber can hold only around ~ 4.5 ml of blood (Fig 1a, 1b). A siphon system was used maintain blood meal temperature (~37 °C) by supplying preheated hot water (40 °C) from a big (~ 5 liters) plastic container to the heating chamber by connecting through an inlet tube. Supplied hot water from the heating chamber was driven out by an outlet tube to another reservoir (R). All these tubes were collected from the saline sets (Fig 1c) used in the hospital. A parafilm membrane of 6 cm x 6 cm dimension (Parafilm “M”, Pechiney Plastic Packing, Chicago, IL. 60631) was stretched approximately twice its size until it became translucent. The outside of the parafilm membrane was rubbed on the human skin to improve the attractiveness of mosquitoes to it. Blood (~ 4.5 ml, ~37 °C) was placed on the lower surface of the heating chamber and the human skin-rubbed parafilm membrane was attached to cover the surface by placing it over the blood chamber, keeping the rubbed-side outward (Fig 1f). The parafilm membrane was fixed firmly on the device with a white teflon tape and rubber bands (Fig 1d). The assembled

apparatus was upside down and fixed with a retort stand (Fig 1e,i) for offering blood meal to the female mosquitoes that confined to a plastic cup covered with fine mesh mosquito net (Fig 2). Preheated hot water (40 °C) collected from a hot water bath was poured into the plastic container placed minimum one foot above of the feeding device (Fig 1g). The flow of hot water from the plastic container to the heating chamber was created by a syringe (Fig 1j) by connecting it with the inlet tube. Hot water from the plastic container passed out by the outlet tube to the reservoir (Fig 1k). Hot water temperature was checked from time to time by a thermometer. Every time the plastic container was filled with pre-heated hot water if it was finished.

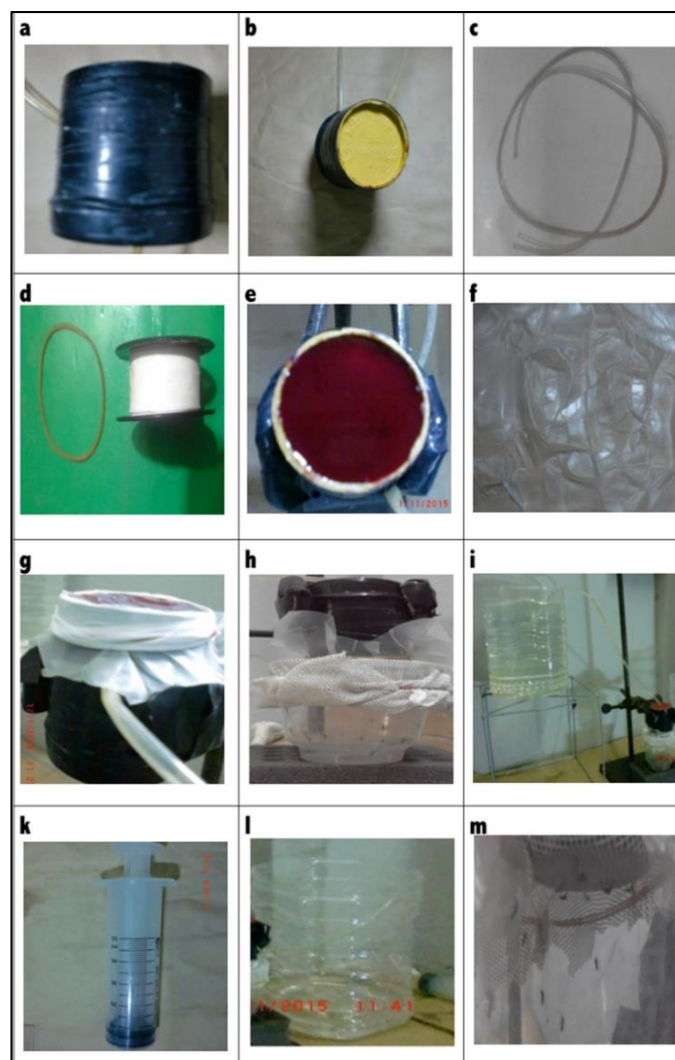


Fig 1: Materials and assembly of the blood feeding device. a. A two-chambered feeding container having a heating chamber (upper part, a capacity of ~ 60 ml water) and blood chamber, (lower part, capacity ~ 5 ml of blood), b. Bottom of the heating chamber where blood meal is poured, c. Disposable saline tube, d. Teflon tape and rubber band, e. Parafilm membrane (6 cm x 6 cm), f. ~ 4.5 ml blood poured on the bottom of the heating chamber, g. Parafilm membrane, teflon tape, and rubber band assembled together, h. upside down feeding container offered to mosquitoes confined to plastic cup covered with mesh mosquito net, i. plastic container with hotwater (40 °C) connected with the heating chamber by an inlet tube, j. Syringe too create a water flow from the plastic container to the heating chamber, k. Water reservoir connected to heating chamber with an outlet tube kept below the level of feeding container, l. Ongoing blood feeding through the device.

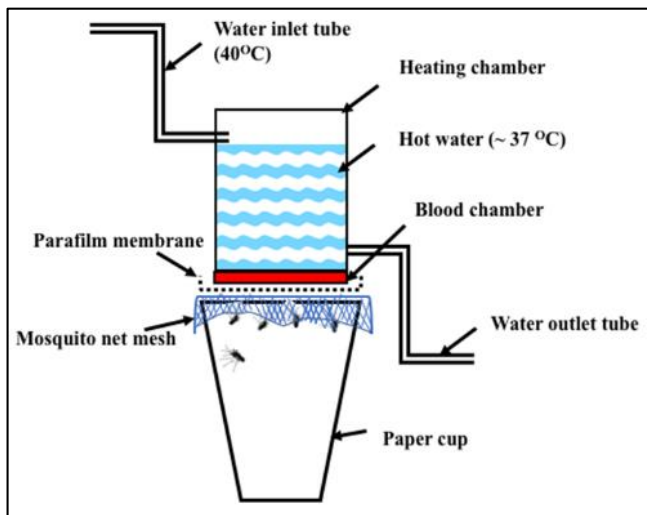


Fig 2: Schematic drawing of the two-chambered blood feeding device offered to *Ae. Aegypti* female mosquitoes. (a) Water (40°C) inlet tube (IT), (b) Parafilm membrane, (c) Mosquito net mesh (d) Heating chamber (HC), (e) Hot water (~ 37°C) (f) blood chamber (BC), (g) Water outlet tube (OT), (h) paper cup with mosquitoes covered with mosquito net mesh

Rearing and use of *Aedes aegypti*

The colony of *Aedes aegypti* mosquitoes including larvae, pupae and adults were maintained according to the procedure described by Foggie *et al.* 2009 [18]. Larvae were fed on finely powdered biscuit crumbs; female and male adult mosquitoes were housed together with mesh screening net and allow them for mating. A piece of cotton ball soaked with 10% sugar solution was placed in a small petri dish was supplied daily to the adult *Aedes aegypti* mosquitoes as food. At the 4th day of age, 25 female *Aedes aegypti* mosquitoes were separated from rearing cage and kept in a plastic cup covered with fine mesh mosquito net and starved for 24 hours. On the following day, the mosquitoes were allowed to feed on either on the feeding device or natural feeding using live pigeons as natural source as used in other studies [19-20]. After one hour of blood feeding, either through the membrane or natural feeding, the fully engorged females were counted, recorded and separated in two mosquito cages and maintained on 10% sucrose solution. The same experiments were repeated 5 times.

Preparing blood for membrane feeding with or without Dengue virus

Non-infectious blood meals were prepared from healthy volunteers by the collection of fresh blood using vacutainer tubes containing Ethylenediamine Tetra Acetic Acid (EDTA) just before it was allowed to feed mosquitoes. By pipetting, the collected blood was mixed well and poured into the lower surface of the heating chamber. For preparing blood meal with Dengue virus, 4 ml of O +ve human blood was collected from a volunteer and mixed with a small amount of plasma positive for Dengue viruses RNA (either Dengue virus serotype 1 or 2 virus), so that it reaches $5 \log_{10}$ RNA copies/ml. It was ensured beforehand that the blood group of both volunteer and virus RNA positive patient was same. The blood meal mixture was prepared 5 minutes before feeding time using a pipette and afterward, the feeding device was assembled as described above to offer the meal to mosquitoes. Experiments with each type of the Dengue virus serotype were performed three times.

Procedure of mosquito Infection

Five-day-old adult *Aedes aegypti* mosquitoes (30 for each experiment) were allowed to feed on that infectious blood meal through the artificial device for 1 hour. After feeding, fully engorged mosquitoes were selected and were reared in cages for 14 days at room temperature. By assuring proper safety precaution, the newly developed feeding device, filled with the blood meal was offered to the female mosquitoes. Starved mosquitoes that were kept within a cup, were placed under the feeder while ensuring that the para film membrane of the small chamber of the device was in contact with the mesh netting fitted to the top of the cup.

Natural Feeding

For natural feeding, live pigeons (aged 3-4 weeks old) were used to provide the same accessible surface area (~ 4 cm) for feeding in every test. The wings of pigeon were tied carefully and feathers were removed from its breast region (3.8 cm x 3.8 cm). The pigeon was kept into a small metal cage and put it in a big cage (made of fine mesh mosquito net) for mosquito feeding. Afterward, a mosquito containing cup was set carefully under the small cage containing pigeon.

Determination of Blood-Feeding Rate, Fecundity, and Hatchability

In the present study, Blood Feeding Rate (BFR) was calculated as the percentage of mosquitoes taken blood-meal in one test. Only fully engorged mosquitoes were counted and recorded while those mosquitoes failed to engorge were removed and discarded accordingly. To determine the fecundity of *Ae. aegypti*, only engorged mosquitoes from two different feeding groups were transferred into two separate acrylic cages (20X20X30 cm) for egg laying [21]. Four paper cups were placed in each cage with wet blotting paper. The female mosquitoes lay eggs within 72 hours later of blood-meal and the number of eggs was recorded for up to seven days to determined fecundity [22]. The eggs were air-dried for five days and counted under a stereo-microscope and were stored in separate glass jars with desecrators in room temperature where it was free of mite and ants. The formula used to calculate the fecundity rate as follows [23].

Fecundity Rate = the total number of eggs laid / the total number of gravid females.

The hatchability of blood-fed engorged female mosquitoes was considered as the percentage of eggs hatched from the total number of eggs collected in 4 cups placed in each cage. To observe the hatching rate, after 30 days of laying eggs [24], all eggs gathered from two types of feeding methods were immersed in a plastic rearing trays with 500 ml of rainwater and observed for mosquito larvae. The number of larvae hatched was counted up to 7 days.

Detection of Dengue virus in *Aedes aegypti* mosquitoes

After 14 days of ingestion of infectious blood meal i.e. the extrinsic incubation period (EPI) [25], the surviving female mosquitoes in each trial were killed by cold shock [26] and pooled in a sterile micro centrifuge tube (not less than 9 mosquitoes/pool). The pooled mosquitoes were homogenized using plastic grinders in 300 μ l of lysis buffer and centrifuged at 3000/rpm for 15minutes at 4 °C and the supernatant was collected for extraction of total Ribo-Nucleic Acid (RNA). Viral RNA was extracted from the mosquitoes using Viral Nucleic Acid Extraction Kit-II (Geneaid, New Taipei City,

Taiwan) according to the manufacturer's instructions with a few modifications. Total RNA concentration was measured in ng/ μ l by spectrophotometer (Nanodrop 2000/2000C, Thermo Scientific, USA). Dengue viral RNA was detected using DENV Real Time-Polymerase Chain Reaction (RT-PCR) kit (oasig OneStep-150 qRT-PCR, Primerdesign Ltd. UK) in a single step procedure as previously described [27].

Statistical Analysis

The data were prepared and organized in MS Excel spread and analyzed using Statistical Package for Social Science (SPSS) version 22. Student's t-test was performed to determine the differences between the mean of blood feeding rate, fecundity and hatchability of the two feeding methods. P values of less than 0.05 were regarded as statistically significant. All the rates were expressed in percentage.

Table 1: Comparison of Blood Feeding Rate (BFR), Fecundity and Hatchability of female *Ae. aegypti* fed on Artificial Membrane (M) and Natural (N) blood- feeding Device.

Parameters	Mode of infection	Number of experiments					Total (%)	Mean	*p value
		1	2	3	4	5			
		Feeding rate fed/n (%)							
BFR (%)	M	18/25 (72)	18/25 (72)	18/25 (72)	19/25 (76)	20/25 (80)	93/12 (74.4)	18.60	>0.5
	N	20/25 (80)	19/25 (76)	19/25 (76)	20/25 (80)	18/25 (72)	96/12 (76.8)	19.20	
Fecundity	M	1762 (97.8)	1683 (93.5)	1693 (94.05)	1811 (95.31)	1598 (79.9)	8807 (92)	1761.40	>0.05
	N	1917 (95.85)	1809 (95.24)	1796 (94.52)	1845 (92.25)	1778 (98.77)	9145 (95)	1829.00	
Hatchability	M	1513 (85.86)	1486 (88.29)	1562 (92.26)	1573 (86.85)	1581 (98.93)	7715 (90.26)	2571.67	>0.5
	N	1757 (91.65)	1708 (94.41)	1663 (92.59)	1685 (91.32)	1657 (93.19)	847 (92.61)	2823.33	

*Student's t-test was performed where p value < 0.5 indicates significant.

Dengue virus transmission in *Aedes aegypti*

After 14 days of post feeding, 167 *Aedes aegypti* mosquitoes were organized in 16 pools and processed for detection of Dengue viral RNA. Out of the 16 mosquito pools, Dengue

Results

Blood feeding Rate, Fecundity & Hatchability

To evaluate feeding efficiency, the total number of engorged female mosquitoes fed by the newly developed feeding device was compared with the female mosquitoes fed on the pigeons (Table-1). The blood feeding rate of *Aedes aegypti* fed on membrane device achieved an average of 74.4%, which was almost same (76.8%) as the natural feeding method. The average fecundity of the feeding device and natural feeder were 95% and 92% respectively. The hatchability rate of *Aedes aegypti* through the device developed in the present study and natural feeding method through pigeon were respectively 90% and 93%. There was no difference observed in the mean of feeding rate, fecundity and mean hatchability rate between two feeding methods (p > 0.05).

virus RNA was detected in 8 (50%) pools (Table-2). Among them, 4 mosquito pools were positive for each kind of serotype (Table-2).

Table 2: Transmission of Dengue viruses -1 & 2 in *Aedes aegypti* female mosquitoes through artificially fed infectious blood.

Experiment No.	Dengue Virus serotype	Total Mosquitoes	No. of Pool +Ve for Dengue virus/Total pool for experiments	% of pool +Ve for Dengue virus
1	1	23	0/2	0%
2		22	2/2	100%
3		20	1/2	50%
4		19	1/2	50%
5	2	20	0/2	0%
6		23	1/2	50%
7		18	1/2	50%
8		19	2/2	100%

Discussion

In the present study, a cheap, simplified blood feeding device was developed for *Aedes aegypti* mosquitoes using household materials and parafilm membrane with human odor along with a siphon system of hot water. At the initial stage, the BFR of this device was examined to observe the effectiveness of the device, and found similar to feeding rate observed in natural feeding procedure. The BFR of *Aedes* mosquitoes differs in different artificial membrane feeding devices. It was 51.3% for *Aedes aegypti* in Glytube feeder [28] and 30-32% for *Aedes albopictus* where parafilm membrane [29] was used. The rate for *Aedes albopictus* fed on human blood was 50% using chicken skin and was 57% using cattle skin [30]. Comparing all these previous results, the feeding rate achieved through this parafilm membrane (72-80%) based blood feeding device is sufficient to maintain colonies of laboratory mosquito strains alternative of the natural feeder and serve as a useful blood meal source. These findings show that all the factors required

to attract mosquitoes to this device were maintained. It is assumed that by preparing a tighten para film membrane with the odor of human skin and hot water syphon system which mimic the natural condition of blood feeding. Besides these, warming the feeding blood to around 37°C which is the normal human body temperature increases the permeability of the para film attached to the apparatus and leads to subsequent leakage of blood [31]. However, feeding through this device shows a negligible reduction in fecundity when compared to natural feeding. This trend was reported before by other researchers also [24]. To feed the *Aedes aegypti* through an artificial feeding device, human blood is better for oviposition while chicken and pig were the least favorable [32].

Previous studies have shown that mosquito's fecundity is affected by mosquito species [33], body size [34], host [35] and volume of ingested blood meal [36]. Blood feeding triggers egg development in *Aedes aegypti* mosquitoes. Each *Aedes* mosquito produces less than 100 eggs in every gonotrophic

cycle [37]. Using this device, there was no difference in fecundity observed in mosquitoes of the two study groups which shows that this feeding device is usable for feeding mosquitoes in laboratory experiments. Hatchability is another important indicator to observe the efficiency of an artificial feeding device. The hatchability rate of *Aedes aegypti* mosquitoes through the artificial membrane feeders vary from 90.8% to 99.33% [32, 38] and the rate observed through this artificial feeding device was within this range. The mosquito feeding position in the developed blood-feeding device is vertical which may be one reason for an increased in the number of engorged females [39].

Another major aim of the study was to observe the transmissibility of Dengue virus in *Ae. aegypti* mosquitoes through this artificial blood feeding device. After 14 days of blood feeding, Dengue virus RNA was detected in 50% pools (8/16) of the infected *Aedes aegypti*. Though the infection rate observed using the feeding device was lower than the rate observed in direct skin feeding of *Aedes aegypti* on Dengue infected patients [16] these experiments showed good concordance with other available artificial devices [16, 31].

The parafilm membrane used in the device is a cheap and one-time use membrane and discarded after each experiment as infectious waste. This device is made of easily available home-made vessels therefore the feeding part of the device need not to be reused and can be destroyed following the usual laboratory procedure. In this research work, we were unable to compare the newly developed device with the commercially available feeding devices that are used in laboratories and we pooled the mosquitoes in each experiment instead of studying every individual mosquito for detection of Dengue virus after infected blood meal. Apart from these limitations, this research work demonstrates that this newly developed device is cheap, low cost as most of the components needed to construct the device was collected from common household materials and available in laboratories. Therefore, it is assumed that after little improvisation, this device would be a better replacement for other expensive devices used in laboratories and could be used in research for mosquito- borne diseases.

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