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Short communication

Comparative Analyses on Synthetic Membranes for Artificial Blood Feeding of *Aedes aegypti* using Digital Thermo Mosquito Blood Feeder (DITMOF)

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ABSTRACT

The use of live animal to blood feeding mosquito colony is proven to be expensive and inconvenient. As an alternative, artificial feeding (AF) is used to rear mosquito colony. The use of synthetic membrane in AF provided a more convenient method as compared to natural membrane which require extensive preparation. In this study, three synthetic membranes were compared (Parafilm-M, Polytetrafluoroethylene tape or PTFE tape and collagen sausage casing) to blood feeding *Aedes aegypti*. The membranes were incorporated with our in-house developed device named as Digital Thermo Mosquito Blood Feeder (DITMOF) to heat cattle blood for mosquito feeding. Results showed that PTFE tape recorded the highest blood feeding rate (95.00% ± 1.67%) with significant mean difference (p <0.001) as compared to both Parafilm-M (72.00% ± 2.60%) and collagen sausage casing (71.50% ± 3.50%). However, there was no difference in term of fecundity for mosquito feed with all three membranes tested (p=0.292). In conclusion, PTFE tape

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should be considered as the preferred membrane to blood feeding *Ae. aegypti*. Furthermore, this artificial blood feeding system, DITMOF successfully feed *Ae. aegypti* conveniently and effectively, thus should be further tested to feed other mosquito species.

Keywords: Aedes aegypti, artificial feeding, blood feeding device, collagen sausage casing, DITMOF, Parafilm-M, PTFE tape, synthetic membrane

INTRODUCTION

Mosquito colony in the laboratory is essential for the study of vector-parasite interactions, insecticide susceptibility, mosquito behaviour, attractant or repellent cues and others. Generally, live animals such as rat, mice and guinea pig or even human volunteer are utilized to blood feed the mosquito colony (Ross et al., 2019). This technique known as direct feeding (DF) has certain advantages such as mosquito blood feeding response that is based on natural signals (WHO, 1995). However, rearing mosquito with this technique has been proven to be costly, inconvenient, and ethically questionable (Nasirian & Ladonni, 2006). Besides, the use of laboratory animals without any quality-assured care and treatment may affect the experimental results (Baumans et al., 1993). Systematic allergic reactions among these animals to mosquito saliva were also reported (Peng et al., 2004). As a result, an alternative mosquito blood feeding technique with no dependency on live animal is needed to overcome these problems.

Artificial blood feeding (AF) is a technique whereby mosquito can be fed using the combination of blood feeder device and animal blood or blood meal substitutes (BMS) for eggs development. The first AF device was developed in 1900's (Wade, 1976). In 2000's, many blood feeder devices invented had utilized or modified the water bath to heat the blood to achieve the range of human body temperature (Klun et al., 2005). Besides the water bath, several other different heating elements used were ceramic heating element (Deng et al., 2012), preheated glycerol (Costa-da-Silva et al., 2013), preheated metal plate (Gunathilaka et al., 2017) and preheated water (Siria et al., 2018). Commercialized blood feeder is also available such as Hemotek membrane feeding system (Hemotek, Accrington, United Kingdom). This system uses its own heating element and can feed up to six cages simultaneously. However, due to the demand in mosquito research and control, an advanced blood feeder equipped with modern technology such as microprocessor and sensor with features like automated temperature regulation, feeding time and effective membrane are needed to reduce the labour, operating costs and improve the efficacy of mosquito rearing (Romano et al., 2018).

The use of membrane in AF is to provide a skin-like surface for female mosquitoes to land before using its proboscis to penetrate the membrane for blood meal. Generally, two types of membranes used in AF are natural and synthetic membranes. In previous studies, natural membranes such as mouse skin and quail skin were used (Novak et al., 1991). However, the disadvantages of these natural membranes are shorter shelf-life and require longer preparation as compared to easily available synthetic membrane. For synthetic membrane in AF, the Parafilm-M was the most widely used (Gunathilaka et al., 2017). Other popular synthetic membranes such as collagen sausage casing (Luo, 2014), latex condom (Novak et al., 1991) and recently, the polytetrafluoroethylene (PTFE) tape or plumber's tape (Siria et al., 2018) were also tested to explore the best effective membrane to feed mosquitoes. Nonetheless, only few studies that specifically compared blood feeding efficacy among these synthetic membranes (Deng et al., 2012; Luo, 2014).

For AF, the most common type of blood used is bovine or cattle (Finlayson et al., 2015). Gunathilaka et al. (2017) reported that there were no significant differences between *Ae. aegypti* mosquitoes fed with bovine and human blood in terms of fecundity, oviposition rate, and fertility. Another alternative blood source is rabbit blood. However, not all animal blood is suitable for mosquito blood feeding for instance sheep blood, whereby lower feeding rate was observed (Paris et al., 2018). Nonetheless, with the development of blood meal substitute (BMS) product such as SkitoSnack (Gonzales et al., 2018), the dependency on animal's blood could be reduced and AF efficiency would be improved. However, this BMS has not been used extensively as the commercial BMS is not yet available.

Previous studies by Deng et al. (2012) and Pothikasikorn et al. (2010) that compared AF with DF technique found that there was no significant difference in terms of fecundity, survival rate, hatchability, and adult mosquito development to development between these two-feeding techniques. Mosquito attraction to host cues and feeding ability on human were also not affected even after AF was performed for eight generations (Ross et al., 2019). Many other studies conducted also utilized mosquitoes that was reared artificially (Paris et al., 2018).

Recently, new mosquito control techniques such as Sterile Insect Technique (SIT) and Incompatible Insect Technique (IIT) that require the release of large number of mosquitoes to suppress the wild strain mosquito populations which could effectively carry dengue viruses (Zhang et al., 2017). These SIT or IIT mosquitoes are usually produced in the mosquito mass rearing facilities whereby millions of mosquitoes are reared. Therefore, these rearing facilities need modern and effective rearing equipment to rear the large-scale mosquitoes efficiently. Nevertheless, only a few new equipment that have been proposed for the use in the mosquito mass rearing facilities (Balestrino et al., 2012). This leads to our invention of DITMOF which is foreseen to have large potential to support such purpose. Nonetheless, this device requires the incorporation of suitable synthetic membrane for mosquito blood feeding. Thus, in this study, we aimed to compare the effectiveness of three types of synthetic membranes heated using DITMOF for blood feeding of *Ae. aegypti*. It is hoped that findings of this study will help to indicate the effective membrane for *Ae. aegypti* AF and overall improve the mosquito rearing efficiency in the laboratories.

MATERIALS AND METHODS

Ethics Statement

Neither humans nor laboratory animals were involved in this study. The procedure of using cattle blood to blood feeding *Ae. aegypti* colony was approved by Animal Ethic Committee, National University of Malaysia with UKMAEC approval number: PARST/PP/2017/AISHAH/22-NOV./885-NOV.-2017-MAC.-2019-AR-CAT2 on 22nd November 2017. The permission to perform this study was granted by Medical Research Committee, Faculty of Medicine, National University of Malaysia with project code number: FF-2017-492 on 21st December 2017.

Mosquitoes and Eggs

Laboratory strain of *Ae. aegypti* eggs batch F1071 was obtained from the Institute for Medical Research (IMR), Ministry of Health, Malaysia. *Aedes aegypti* eggs were soaked in dechlorinated water to induce hatching and larvae were fed with both TetraMin fish food and beef liver powder at 1:1 ratio. The rearing water was replaced daily to prevent any scum formation on the water surface. Pupae were transferred to rearing cage for adult emergence and adults were fed with 10% sugar solution with added 1% Vitamin B complex. Both female and male *Ae. aegypti* mosquitoes were left for 3-5 days in adult rearing cage at 1:1 ratio to ensure mosquito mating. The insectary temperature was maintained at 25 ± 3ºC, 80% ± 10% humidity with natural photoperiod.

Type of Blood and DITMOF Device

Cattle blood used in this study was obtained from the Jasin Abattoir Complex, Department of Veterinary Services, Melaka, Malaysia. The blood was mixed with anticoagulant CPDA-1 at 1:7 ratio (anticoagulant: blood ratio) and was put inside a cooler box with ice packs for transportation before being stored in a chiller at 1 to 6ºC. This chilled citrated cattle blood could be used for a maximum 35 days (Rudmann, 2005).

The in-house developed automated mosquito blood feeder, Digital Thermo Mosquito Blood Feeder, or DITMOF (patent pending) was used to heat the cattle blood to be used in the AF of *Ae. aegypti*. DITMOF device is consisted of a digital thermo controller (Shinko BCS1), K thermocouple sensor (VITAR standard thermocouple sensor PS-C-1M), stainless steel casing (128 mm x 128 mm x 198 mm), strip heater (VITAR LBAA26 240V/450W), power plug (UK 13A), blood feeder reservoir (55 mm diameter, 5 mm depth) and external digital thermometer.

Membrane Test and Blood Feeding Rate

Three types of membranes that were selected for evaluation were Parafilm-M (10.2 cm x 38.1 m, Bemis, USA), Polytetrafluoroethylene (PTFE) tape or plumber's tape (RS PRO

White, 12 mm × 12 m × 0.075 mm, Malaysia) and collagen sausage casing (32 mm, The SausageMaker Inc, Buffalo New York, USA). On the day of blood feeding, all three membranes were cut in 9 cm in length and subsequently, each membrane was attached on top of the blood feeder reservoir as shown in Figure 1. Excess membranes at the edge of the blood feeder reservoir were cut off. Stretched PTFE tape and Parafilm-M membranes were self-adhesive thus it can be attached directly on blood feeder reservoir on its own. To prevent Parafilm-M from snapping during blood feeding, it was carefully stretched uniformly and was measured not to exceed more than twice its original size. However, for collagen sausage casing it was boiled under pressure for three minutes, washed twice with tap water to remove any excess oil and the rubber band was used to attach the collagen sausage casing onto the blood feeder reservoir as shown in Figure 1c.

Next, 30 ml of chilled blood in three separate universal bottles (10 ml each) were preheated at 40ºC in warm water for 5 minutes. Subsequently, for each blood feeder reservoir attached with Parafilm-M, PTFE tape and collagen sausage casing, 10 ml of preheated blood was transferred into each blood feeder reservoir. Next, to attach or secure the blood feeding reservoir to DITMOF heating surface, each blood feeding reservoir was wrapped with thin layer of Parafilm-M at the bottom. This thin Parafilm-M layer acted as a bonding substance between the blood feeder reservoir and DITMOF heating surface in which it will become sticky when the heat is applied. Constant heating during blood feeding ensures blood feeder reservoir to stick on DITMOF heating surface thus maintaining the desired blood temperature as has been set on the DITMOF digital controller. To ensure constant temperature of DITMOF heating surface during entire feeding time, 30-minute measurement of DITMOF's heating surface using infrared thermometer yielded a constant temperature ranging ± 0.1°C after targeted temperature was achieved. However, based on three confirmatory testing at 0, 15 and 30 minutes for each membrane attached to blood feeder reservoir, different temperature range had to be set on DITMOF digital thermo

(a) (b) (c)

Figure 1. Attachment of membrane to blood feeder reservoir a) Parafilm-M; b) PTFE tape; c) Collagen sausage casing. Rubber band was used to attach collagen sausage casing to blood feeder reservoir.

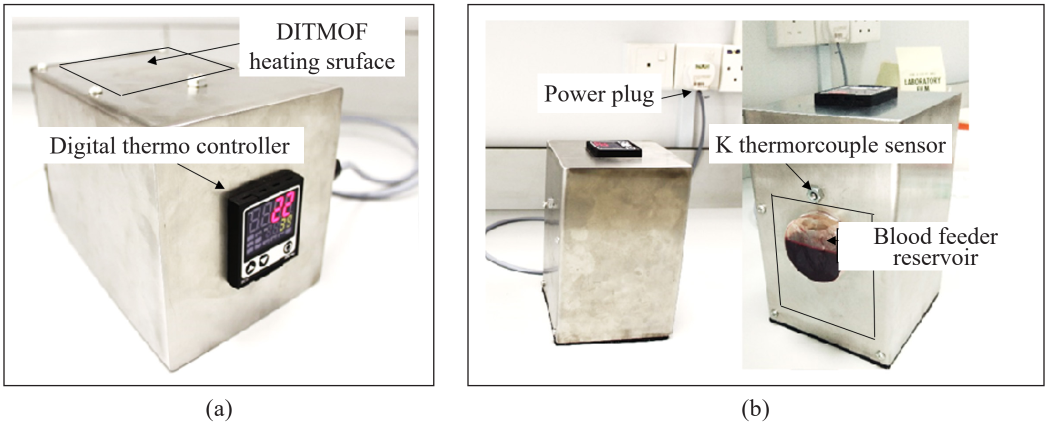
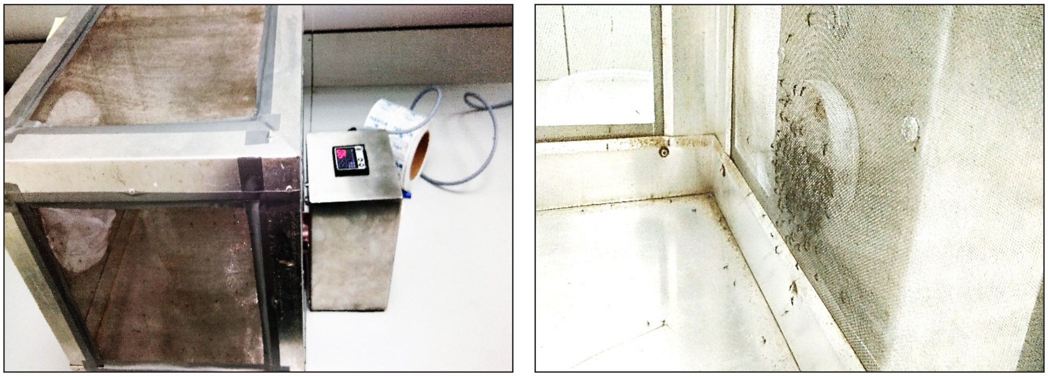


Figure 2. Digital Thermo Mosquito Blood Feeder (DITMOF) in: (a) horizontal position; and (b) vertical position (feeding position)

controller for each membrane. For PTFE tape and collagen sausage casing, temperature had to be set at 54ºC while for Parafilm-M it was set at 40ºC to ensure that the blood temperature in the blood feeder reservoir could be maintained at 35ºC - 37ºC range. The mean temperature of blood in Parafilm-M was 36.2ºC, PTFE tape was 35.9ºC and collagen sausage casing was 35.9ºC (ANOVA, p= 0.121). It was presumed that different temperature settings between these membranes were due to the membrane ability to retain the heat. The DITMOF device and its vertical feeding position are shown in Figure 2.

The mosquitoes used in this test were 3-5 days old mated female *Ae. aegypti* which were previously starved for 24 hours prior to blood feeding (Siria et al., 2018). For each replicate, 20 mosquitoes were put inside the test cage (20 cm × 20 cm × 20 cm). The cage was put side-by-side to DITMOF position for blood feeding process as shown in Figure 3. Total blood feeding time for each replicate was 30 minutes and black cloth was used to cover the test cage throughout the blood feeding period to minimize the interference. During the first one-minute of blood feeding, human breath was expelled near blood feeder reservoir to enhance the mosquito response. Number of mosquitoes landed on membrane after 1−minute human breath expelled was also recorded. At 15 minutes interval, fully engorged females were taken out and the remaining unfed mosquitoes were offered with blood feeding for another 15 minutes. After 30 minutes completed, total number of fully engorged females were calculated and analysed. Fully engorged mosquito was determined by visual inspection of fully distended or stretched mosquito abdomen (Ciota et al., 2011). Blood feeding rate was calculated as number of blood-fed females / numbers of mosquito tested × 100%. This method was repeated for all membranes type with 10 replicates performed for each membrane. The test was conducted at the same time during the day (1400 hours-1600 hours) for 10 consecutive days.



(a) (b)

Figure 3. Mosquito artificial blood feeding using DITMOF device: (a) DITMOF and test cage at side-by-side position; and (b) *Ae. aegypti* mosquitoes were having blood meal.

Fecundity Rate

To measure fecundity, 10 mosquitoes three days post blood feeding were used for each replicate. Mosquitoes were put inside rearing cage where black paper cup containing 150 ml dechlorinated adapted for oviposition was added. This black paper cup was added with cone-shape filter paper to provide suitable oviposition sites for *Ae. aegypti*. After 48 hours exposure, total eggs accumulated on filter paper were calculated using stereo microscope. 10 replicates were performed for every membrane. Fecundity in this study was defined as total number of eggs collected per 10 mosquitoes.

Statistical Analysis

To evaluate the number of *Ae. aegypti* landed on blood feeder reservoir, blood feeding rate and fecundity using tested membranes, the parametric one-way analysis of variance (ANOVA) and Bonferroni post-hoc tests were used at significance level of $p<0.05$, given that the data collected was normally distributed. Data normality testing was performed using the Shapiro-Wilk and Kolmogorov-Smirnov test. All statistical analyses were completed using the SPSS version 24 from IBM.

RESULTS

In terms of the membrane preparation for the AF, both PTFE tape and Parafilm-M were easier to be handled as compared to the collagen sausage casing. The PTFE tape and Parafilm-M are self-adhesive especially when being stretched out and could be easily attached to the blood feeder reservoir. In contrast, the attachment of the collagen sausage casing was trickier as the use of the rubber band could cause damage onto the collagen sausage casing and it could also be easily slipped out from the blood feeder reservoir especially when it is wet. However, for all three membranes tested, no blood leakage was observed during the whole feeding period using DITMOF device.

The number of mosquitoes landed on the blood feeder reservoir after 1−minute of feeding duration showed that the highest mean was recorded for PTFE tape at 15.20 ± 0.39, followed by Parafilm-M at 14.30 ± 0.52 and collagen sausage casing at 13.9 ± 0.46. However, ANOVA analysis showed that there was no significant mean difference of number of landed mosquitoes among tested membrane with p value of 0.140. For blood feeding performance, after the first 15-minutes of blood feeding completed, PTFE tape membrane recorded the highest *Ae. aegypti* blood feeding rate with 90.00% ± 2.47%, followed by Parafilm-M (56.00% ± 2.21%) and collagen sausage casing (54.50% ± 3.11%). Analysis of variance (ANOVA) with post-hoc Bonferroni test showed significant difference for the use of PTFE tape as compared to the Parafilm-M and collagen sausage casing with both p-values were <0.001. However, the difference between Parafilm-M and collagen sausage casing was not significant with a p-value of 1.000. Subsequently, after the full blood feeding test was completed in 30 minutes, the similar pattern was observed. The use of PTFE tape recorded the highest *Ae. aegypti* blood feeding rate with 95.00% ± 1.67%, followed by the Parafilm-M (72.00% ± 2.60%) and collagen sausage casing (71.50% ± 3.50%). Only PTFE tape membrane showed a significant difference as compared to both Parafilm-M and collagen sausage casing with p value of <0.001. The utilization of Parafilm-M and collagen sausage casing were not significantly different to each other with p-value of 1.000. Comparison of the mean mosquito landed after 1−minute of blood feeding duration and the blood feeding rate at 15−and 30−minutes interval for three tested membranes are shown in Table 1.

For fecundity among mosquitoes that had been artificially fed using different type of membranes, the highest mean was recorded using Parafilm-M at 532.10 ± 22.67 followed by PTFE tape at 502.90 ± 19.00 and collagen sausage casing at 487.20 ± 18.25. However,

Table 1
Comparison of the mean mosquito landed after 1−minute of blood feeding duration and the blood feeding rate at 15- and 30-minutes interval for three tested membranes

Membrane type	Mean mosquitoes landed after 1 minute (n=20) ± SEM	Blood feeding duration	Blood feeding rate (%) ± SEM
Parafilm-M	14.30[a] ± 0.52	15 minutes	56.00[a] ± 2.21
		30 minutes	72.00[a] ± 2.60
PTFE tape	15.20[a] ± 0.39	15 minutes	90.00[b] ± 2.47
		30 minutes	95.00[b]± 1.67
Collagen sausage casing	13.90[a] ± 0.46	15 minutes	54.50[a] ± 3.11
		30 minutes	71.50[a] ± 3.50

*Values with the same alphabet are not significantly difference at p level 0.05 while values with different alphabets are significantly different.

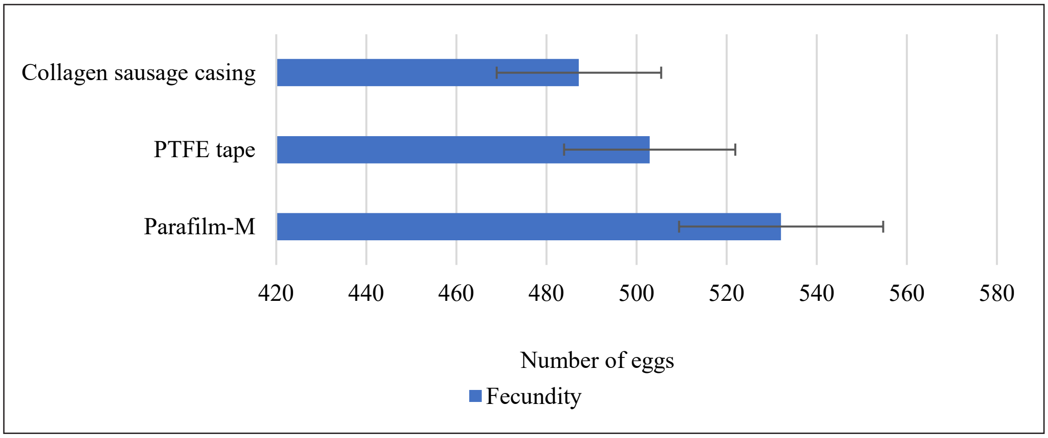


Figure 4. Fecundity of *Ae. aegypti* mosquitoes that had been artificially fed using three tested membranes

there was no significant mean difference among the three tested membranes as determined by one-way ANOVA ($F (2,27) = 1.289$, $p=0.292$). Figure 4 shows the fecundity of *Ae. aegypti* mosquitoes that were blood fed using the three tested membranes.

DISCUSSION

Choosing the correct type of membrane is essential to develop a mosquito blood feeder that can feed the mosquitoes effectively. In this study, we found that the PTFE tape was the most effective synthetic membrane for *Ae. aegypti* blood feeding. As for the Parafilm-M and collagen sausage casing, acceptable feeding rates (>70%) were observed; with no significant difference seen between both membranes. These findings are in line with Luo (2014) who also compared the blood feeding rate of *Ae. aegypti* using the Parafilm-M and collagen sausage casing.

There is a strong indication that membrane thickness could affect *Ae. aegypti* blood feeding ability (Luo, 2014). The thickness of PTFE tape used in this study was rated at 0.075 mm while the Parafilm-M was thicker at 0.127 mm. The PTFE tape was stretched out for more than twice its size resulting in the final membrane thickness of less than 0.038 mm. For Parafilm-M it was stretched out about twice its size resulting in the final membrane thickness approximately 0.064 mm. This step was performed carefully to prevent over-stretching of Parafilm-M. Over-stretched Parafilm-M could develop weak spot on its surface and increase the risk of rupture especially when heating. At 0.064 mm thickness, it was stretched far from its braking point at 0.025 mm (Luo, 2014). Additionally, other researcher that used Parafilm-M in AF also stretched about twice its original size (Nasirian & Ladonni, 2006). As the boiled collagen sausage casing, the thickness was rated at 0.073 ± 0.004 mm (Luo, 2014). Collagen sausage casing could not be stretched-out; thus, it was categorized as the thickest membrane among the three.

It was also postulated that the membrane pore size influences the blood feeding efficacy of *Ae. aegypti.* Bigger pore sizes enhanced the mosquito proboscis penetration. The type of membrane used would also affect the on piercing and probing of mosquito blood feeding (Novak et al., 1991; Ross et al., 2019). Direct membrane pore measurement was not performed in this study. However, PTFE tape pore size was reported to have an average of 500 nm and this pore size may increase if the tape is stretched out (Van der Linden, 1983). In contrast, the pore size of a similar non-stretchable collagen sausage casing has an average of 48.2 nm (Ledesma et al., 2015) while the Parafilm-M is a non-porous wax-modified polyolefin film.

The blood feeding rates for the 15-minute and 30-minute blood feeding were higher for the PTFE tape as compared to the Parafilm-M and collagen sausage casing. However, there was no significant mean difference of the number of mosquitoes landed on all three membranes at first minute of the blood feeding duration (p=0.140). These findings indicated that the attraction of mated female mosquitoes towards all membrane attached to blood feeder reservoirs were almost similar. Nonetheless, mosquito feed on PTFE tape membrane recorded a higher and significant blood feeding rate as compared to the others.

Besides being more effective for *Ae. aegypti* blood feeding, the PTFE tape membrane is also more heat resistant, with the maximum operating temperature rated at 260ºC (RSPRO, 2020). For collagen sausage casing, the boiling of this synthetic membrane in water will not change its basic microstructure and thickness (Barbut, 2010). In contrast, the Parafilm-M becomes soft and sticky at 54ºC and completely melt at 100ºC (AMCOR, 2019). When the heat was used to warm the blood, the Parafilm-M membrane is more prone to disintegrate and cause the blood leakage especially if it was used for a longer period. In these circumstances, the PTFE tape and collagen sausage casing are more suitable to be used as the blood feeding membrane in AF.

In term of fecundity, there was no significant mean difference of eggs produced among all the tested membranes although the slightly higher mean was seen using Parafilm-M. The use of the same cattle blood source as a standard blood for mosquito feeding in this study could contributed to these non-significant differences. There were reports indicate that different blood types using in AF could have effects on fecundity. For example, Gunathilaka et al. (2017) reported that there was higher and significant mean difference of *Ae. aegypti* that were feed using bovine blood compared to chicken blood. However, our study shows that the use three different synthetic membranes tested have minimal effect to mosquito fecundity.

Ae. aegypti mosquitoes used in the testing has successfully been maintained for one year and a half using DITMOF and Parafilm-M membrane combination. Thus, it is suggested that DITMOF as a proven blood feeder device for rearing *Ae. aegypti* mosquito. The use of DITMOF device in the AF provided a more convenient blood feeding process where constant and precise blood temperature was achieved. In comparison, several other blood feeder devices that are using preheated medium such as glycerol, metal plate or water, require continuous monitoring to maintain a precise and constant feeding temperature (Costa-da-Silva et al., 2013; Gunathilaka et al., 2017; Siria et al., 2018). Furthermore, feeding process using DITMOF does not require modification to the adult cage due to the ready-to-use design and additional feature such as adjustable feeding temperature which could provide a better temperature option for users.

Moreover, up to four blood feeder reservoirs can be attached to DITMOF heating surface at one feeding session to feed many mosquitoes. The DITMOF side-by-side feeding position also minimizes the risk of membrane rupture where less weight and pressure were put on a fragile membrane. This side-by-side feeding setting also ensures that the mosquito blood feeding could be performed in a nose-up vertical position instead of traditional upside-down horizontal position. This nose-up vertical feeding position allows a more normal flow of blood to be achieved and for *Aedes albopictus* species, an increase of 46% blood feeding rate was reported (Lyski et al., 2011). Additionally, mosquito with a heavy abdomen inclines to maintain in nose-up vertical position, thus blood meal offered in upside-down feeding position in most traditional blood feeder device, are less favourable for the blood meal consumption (Bender & Frye, 2009). However, further comparison and tests with current commercial blood feeder should be performed to confirm these advantages in rearing the mosquito colony.

CONCLUSION

In this study, the use of PTFE tape as membrane indicated superior blood feeding performance for *Ae. aegypti* mosquitoes as compared to the Parafilm-M and collagen sausage casing. This membrane should be the preferred synthetic membrane in the AF to feed *Ae. aegypti* mosquitoes. In combination with the effective membrane, the in-house developed automated mosquito blood feeder, DITMOF shows great a potential to be used as a mosquito blood feeder in mass rearing mosquito facilities or small laboratories. Further testing to feed other mosquito species should be conducted using this device. It is hoped that the findings and invention from this current study will help in the enhancement of mosquito production and facilitate the mosquito research and control programmes.

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