The Effect of Anticoagulant in Blood Meal Source on the *Aedes aegypti* Reproductive Ability in Laboratory

Novyan Lusiyana[1], Budi Mulyaningsih[2], Sitti Rahmah Umniyati[2]
[1]Department of Parasitology, Faculty of Medicine, Universitas Islam Indonesia, Yogyakarta, Indonesia;
[2]Department of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia

Corresponding: lusiyananovyan@yahoo.com

ABSTRACT

Introduction: *Aedes aegypti* is one of the major vectors of dengue hemorrhagic fever (DHF) that can be reared in laboratory. Artificial membrane feeding (AMF) assay is used as a simulated host to blood feeding mosquitoes in laboratory.

Objectives: The purpose of this study are to investigate the effect of heater and the most widely used anticoagulant of K_3EDTA, heparin and sodium citrate on blood feeding sucsses, feeding rate, fecundity, hatchability, preadult development and survival rate of *Aedes Aegypti* colonies maintained by AMF system compared to direct human feeding.

Methods: The system consisted of AMF with parafilm membrane which are warmed and not warmed by a waterbath. Human blood samples were used to feed *Aedes aegypti* using AMF. The number of eggs were counted seven days after feeding after mosquitoes laid the eggs. Every eggs were hatched in a 500 mL of rearing glass to evaluate the hatchability and preadult development. Survival rate is evaluated after blood feeding until 30 days. Data was analyzed using one-way ANOVA and paired t-tests and a p value <0.05 considered as significantly difference.

Results: Blood feeding success of *Aedes aegypti* was not significantly differ when offered blood meal using anticoagulant heparin with heater (82.22%) compare to that of control groups (81.67%) (p=0.917). There was a significant difference in feeding rate (p=0.000), fecundity (p=0.000), hatchability (p=0.000) between groups. All results were higher in heparin than K_3EDTA and sodium citrate, but in pre adult development and survival rate K_3EDTA showed better result than that of control, heparin and sodium citrate groups. So this anticoagulant was acceptable for maintenance of laboratory colonies of *Aedes aegypti*.

Conclusion: We conclude that heater can increase the blood feeding sucsses. The K_3EDTA, heparin and sodium citrate can affect the feeding rate, fecundity, hatchability, and preadult development, but do not affect survival rate. Heparin can be used for routine colonization of laboratory strain of *Aedes aegypti* with AMF assay.

Keywords: Anticoagulants, artificial membrane feeding, reproductive ability, survival rate

INTISARI

Pendahuluan: *Aedes aegypti* adalah salah satu vector penting penyakit *dengue hemorrhagic fever* (DHF) yang dapat dikembangbiakkan di dalam laboratorium. Metode *artificial membrane feeding* (AMF) digunakan sebagai inang buatan untuk *blood feeding* nyamuk di laboratorium.

Tujuan: Untuk meneliti efek pemanasan dan penambahan antikoagulan K_3EDTA, heparin and natrium sitrat

pada keberhasilan *blood feeding*, *feeding rate*, fekunditas, kemampuan *hatching*, perkembangan preadult dan *survival rate* koloni *Aedes Aegypti* yang dipelihara dengan sistem AMF dibandingkan dengan pemberian makan langsung pada manusia.

Metode: Sistem ini meliputi AMF dengan membran parafilm baik yang dihangatkan maupun tidak dihangatkan dengan *waterbath*. Sampel darah manusia digunakan untuk memberi makan *Aedes aegypti* dengan menggunakan AMF. Jumlah telur dihitung tujuh hari setelah pemberian makan dan nyamuk meletakkan telurnya. Setiap telur dilakukan *hatching* untuk mengevaluasi kemampuan hatching dan perkembangan preadult. *Survival rate* dievaluasi sampai 30 setelah *blood feeding*. Data dianalisis dengan Uji ANAVA satu jalur dan Uji t berpasangan dengan p <0,05 sebagai batas kemaknaan.

Hasil: Keberhasilan *blood feeding* nyamuk *Aedes aegypti* tidak berbeda secara bermakna pada kelompok yang diberi perlakuan pemberian antikoagulan heparin dan pemananasan (82,22%) dibandingkan dengan kelompok kontrol (81,67%) (p=0,917). Terdapat perbedaan yang bermakna pada *feeding rate* (p=0,000), fekunditas (p=0.000), kemampuan *hatching* (p=0.000) antara semua kelompok. Semua hasil menunjukkan bahwa kelompok heparin lebih tinggi dibanding kelompok K_3EDTA dan natrium sitrat, tetapi perkembangan preadult dan *survival rate* kelompok K_3EDTA lebih baik dibanding dengan kelompok kontrol, heparin dan natrium sitrat. Antikoagulan heparin dapat digunakan untuk memelihara koloni *Aedes aegypti* di dalam laboratorium.

Simpulan: Pemanasan dapat meningkatkan keberhasilan *blood feeding*. Antikoagulan K_3EDTA, heparin dan natrium sitrat dapat mempengaruhi *feeding rate*, fekunditas, kemampuan *hatching* dan perkembangan preadult, tetapi tidak mempengaruhi *survival rate* nyamuk. Heparin dapat digunakan untuk kolonisasi rutin *Aedes aegypti* dalam laboratorium dengan AMF.

Kata Kunci: Antikoagulan, *artificial membrane feeding*, kemampuan reproduktif, *survival rate*

INTRODUCTION

Aedes aegypti is one of the major DHF vectors in tropical and subtropical world regions. This mosquitoes' species is one of the main DHF vectors in the South East Asian countries and is particularly abundant in Indonesia[1].

Adult female *Ae. aegypti* mosquitoes prefer to feed on human blood as evidenced by anthropophylic behaviour[2]. The colonies of mosquitoes in laboratories are often maintained using animals as a blood source[3]. Currently, live animals such as guinea pigs and rodents are more commonly used, however this method is expensive, time consuming, and subject to government regulation and inspection, thereby limiting its use for some laboratory experiments[4]. Most of earlier studies on mosquitoes rearing relied on human hosts or live animal hosts[5]. Laboratory studies of biology and/or mosquito-pathogen interaction largely employ artificial blood delivery system to feed mosquitoes, particularly in virus study where the use of animals is restricted[4]. Blood feeding of female mosquitoes is an essential activity for colonization and maintenance of mosquitoes, which are often required for research on vector-borne diseases[6]. The increasing awareness on animal welfare and stringency in regulation governing the scientific use of animals for research, coupled with the inconvenience of using live animals as blood host, have led to the impetus for the development of a cheap and user friendly artificial membrane blood feeding system for mosquitoes[5].

In nature, a blood meal is directly essential for energy and reproduction, and indirectly responsible for the transmision of arboviruses [7]. An autogenous female mosquitoes require a blood meal to obtain amino acids from erythrocytes and plasma protein digestion to synthesize yolk proteins for egg productions[8]. Blood feeding can result in ingestion of pathogens, which the mosquitoes can transmit to host during subsequent blood feeding[9]. The membrane feeding technique in the laboratory for blood feeding is necessary for colony maintenance and for virus-host investigations[7]. Blood feeding using AMF needs blood especially from human, because human was the host for many mosquitoes. Blood needs anticoagulant for delay the coagulation so the mosquitoes can be fed with blood. Sodium ethylenediamine tetraacetic acid (K_3EDTA), lithium heparin and sodium heparin are anticoagulants frequently used in blood collection, including for AMF[10]. Each anticoagulant has different effect on the coagulation cascade. The K_3EDTA and sodium citrate affects the coagulation cascade by chelating calcium ions that are required for the activation of factor IX in the intrinsic pathway, and factor VII-Ca-Xa complex in the common pathway[11,12,14]. We speculate that after chelation by K_3EDTA and sodium citrate this ion would not be available for the need of the mosquitoes for reproductive cycle. Lithium heparin binds to antithrombin, thereby accelerating the inhibition of proteases (principally factors Xa and IXa) involved in the coagulation cascade[13].

Herein, we aimed to investigate the effect of three common anticoagulants, K_3EDTA, lithium heparin and sodium citrate on blood feeding in *Ae. aegypti* reproductive cycle to determine optimal reared procedures in laboratory. This knowledge is essential for standardization of AMF assays used for routine production of mosquitoes. In addition, there was no substantial study on the long-term effects of such anticoagulant on the feeding rate, fecundity, hatchability, preadult development and survival rate of the mosquitoes.

MATERIALS AND METHODS

Hatching and rearing of mosquitoes

Ae. aegypti mosquitoes were hatched, reared and maintained in the Gadjah Mada University's Insectary located in Yogyakarta, maintained at temperature of 25^0C-28^0C in 70%-80% of relative humidity, and 12:12 hours of light and dark photo period[5,15,16]. Female and male adults were housed together for 5 days for mating in white bucket cages topped with mosquitoes netting, supplied ad libitum with 10% of sugar solution that was placed in the cage as a food source[16]. The females were then separated from males and deprived of carbohydrates for 24 h before feeding experiments and all trials were conducted in six replications.

Preparation of blood sample for AMF assays

Blood samples were added in vaccutainer tubes which contain three different anticoagulants. To assess the effect of anticoagulant on *Ae. aegypti* reproductive cycle after AMF, sample donors used for mosquitoes feeding. Controls were from the same human with direct feeding.

Artificial glass membrane feeder

Membrane parafilm were secures to glass membrane feeders. Immediately after blood

preparation, 1 mL of blood was placed in the AMF device with heater (constant temperature at 37°C) and with no heater for 60 minutes. Blood was then offered to 120 laboratory-reared 5 to7 days old female of *Ae. aegypti* mosquitoes.

Blood feeding success, feeding rate, fecundity, hatchability, pre adult development and survival rate

One day before blood feeding the mosquitoes was pondered to check the weight before feeding. After 1 hour all blood-fed mosquitoes were picked after the blood meal. Each group mosquitoes was pondered and transferred into a paper glass (300 ml) for egg laying. A small wet cotton wool and a piece of filter paper (5.5 cm in diameter) were placed in the bottom of the paper glass which was inverted for the female to deposit their eggs on the on the wet filter. Each paper glass of blood feed mosquito was maintained by a cotton wool fully soaked with 10% of sucrose solution and placed on top. After one week, the eggs laid by the blood fed mosquitoes were air-dried for 7 days and counted under dissected microscope to determine the number of eggs laid per female. Seven days after feeding, mosquitoes were placed in cages to check the survival rate. They were transferred into large cages and continued to be fed with 10% of sucrose solution. Survival rate is the percentage of female mosquitoes that survive 30 days after the first blood meal.

Meanwhile, eggs which were laid by mosquitoes were hatched and being used for hatchability studies. Each egg was distributed in a paper glass (500 mL) of water for hatching. The number of larva hatched was counted daily until 7 days. Hatchability is the percentage of larva hatched per number of eggs for each anticoagulant.

Statistical analysis

All data were collected and analyzed by using analysis of variance (ANOVA) or Kruskal-Wallis tests. A paired sample t test was performed to determine the differences between two feeding methods (heater and no heater). Batches of mosquitoes were assessed using two outcome measures i.e. the geometric mean number of eggs and the proportion per batch. The ANOVA test was used to compare the difference between control, K_3EDTA, heparin and sodium citrate groups. Each test was two sided and evaluate at the 0.05 significance level.

RESULTS AND DISCUSSIONS

Blood meal is required for an autogenously female mosquito to complete a gonotrophic cycle. A total of 720 mosquitoes (4 batches) were count in each anticoagulant assay to estimate the effect of K_3EDTA, heparin and sodium citrate on the blood feeding sucsses, feeding rate, fecundity, hatchability, pre adult development, and survival rate. The presence of K_3EDTA resulted in a lower mean eggs number.

Blood feeding success

Comparison of blood feeding success among control and blood with anticoagulant is presented (Table 1). This table shows the feeding sucsses of *Ae. aegypti* offered with control blood and blood with anticoagulant. Feeding rate of *Ae.aegypti* are 147 (81.67%) in control, 36 (19.99%) using K_3EDTA, 65 (36.11%) using heparin and 15 (8.33%) using sodium citrate in trials without heater. In trials with heater the result are 66 (36.60%) using K_3EDTA, 148 (82.22%) using heparin and 92 (51.11%) using sodium citrate. The feeding success of *Ae. aegypti*

which was given blood feeding with heater was significantly better than without heater, and heparin with heater was the same with control groups (p>0.05).

Table 1. Comparison of number blood feeding success of *Ae.aegypti* fed on human control and blood with anticoagulant

Group	No heater	Heater	p**
K1	147±2.35	147±2.35	
K2	36 ±1.89	66± 2.19	0.021
K3	65± 1.47	148± 3.14	0.000
K4	15± 1.05	92± 3.08	0.000
p*	0.000	0.000	

K1 : Control mosquitoes group with human direct feeding
K2 : K_3EDTA blood feeding group with AMF
K3 : Heparin blood feeding group with AMF
K4 : Sodium citrate blood feeding group with AMF
p* *one way ANOVA*, significant if p<0.05
p** paired sample t test, significant if p<0.05

Blood-searching behavior is governed by two behavioral components, probing and giving. Mosquitoes sense purinergic phagostimulant compounds, such as ATP and ADP, which signal contact between their feeding styles and the blood[17].

Ae. aegypti is a mosquito found world wide. By challenging *Ae.aegypti* with several variables, we attempted to identify strategies for improving feeding success of anticoagulant in blood feeding. Blood feeding success of control group of *Ae. aegypti* is the same with heparin group. Phagostimulants such as ATP, L-lactic acid, natural odor ligands from human skin, visual and olfactory stimuli from host result in varying degrees of success[18,19,20,21,].

Blood feeding success in mosquitoes were influenced by complete body emanations including body odor, heat and moisture in human[22]. This reason can be similar with heater in AMF which is needed to make the blood feeding more success, because the temperature is the same with human body's temperature. Temperature influences the ability of mosquitoes to fed, at the low temperature mosquitoes sucking slower than that at higher temperature[23].

Feeding rates

Ae. aegypti's feeding rates among control and blood with anticoagulant were observed in this study (Table 2). *Ae.aegypti* fed blood in average of 2.45 mg in control, 1.57 mg in K_3EDTA, 1.84 mg in heparin and 1.72 mg in sodium citrate groups. The weight of *Ae. aegypti* fed with anticoagulant were significantly less compare with that of control gropus, but heparin group has higher average of weight than other anticoagulant groups.

Table 2. Comparison of feeding rates between *Ae. Aegypti* fed on human control and blood with anticoagulant

Group	Mean feeding rates (mg)	p*
K1	2.45±0.21	0.000
K2	1.57±0.20	
K3	1.84±0.07	
K4	1.72±0.28	

K1 : Control mosquitoes group with human direct feeding
K2 : K_3EDTA blood feeding group with AMF
K3 : Heparin blood feeding group with AMF
K4 : Sodium citrate blood feeding group with AMF
p* *one way ANOVA*, significant if p<0.05

The volume of blood ingested varies between and within species and is influenced by the gut dimension, duration of feeding and the rate of blood uptake[17]. Imbibing blood depends on insect suck and spit activity (pharyngeals pumps), midgut integrity and capacity (stretch reseptor), gripping ability (intact tarsals), feeding physiology, vertebrate host cues associating visual and olfactory stimuli for host blood feeding and conundrum of environmental factor[8]. The process of blood-feeding in the mosquitoes can be divided into four steps: (1) attraction to the host, (2) probing with the fasicle (3) sucking, and (4) withdrawal of stylets[18]. Mosquitoes' position rate increased with body size[24]. Ingested blood volume is well-established as a crucial parameter of fecundity in many mosquitoes and particularly in several *Aedes sp.* Large blood consumption increased fecundity for yolk synthesis. Small mosquitoes have several disadvantages compared to large one, including decrease survival, and reduce fecundities[6,25]. A higher probability of survival was related to larger body size, and blood meal size and fecundity also improved with size [26].

Previous studies have shown that mosquitoes' fecundity increases with increasing blood meal size. This is confirmed when *Ae. Aegypti* produced significantly more eggs with increases blood meal volume. But, the nutritional status of individual females might influence the fate of small blood meals[27].

Fecundity

This study demonstrated variation in fecundity between trials. Table 3 shows the difference of fecundity of *Ae. Aegypti* between control and blood with anticoagulant groups. There was a significant differences in fecundity among the control and blood anticoagulant groups (p>0.05).

Table 3. Fecundity of *Ae. aegypti* fed on human control and fed through blood with anticoagulant

Group	Mean eggs/mosquitoes	p*	Total number of eggs
K1	60.75±0.22	0.000	8,895 (147)
K2	46.94±0.20		3,013 (66)
K3	53.28±0.08		7,877 (148)
K4	49.90±0.28		4,559 (92)

K1 : Control mosquitoes group with human direct feeding
K2 : K_3EDTA blood feeding group with AMF
K3 : Heparin blood feeding group with AMF
K4 : Sodium citrate blood feeding group with AMF
p* *one way ANOVA*, significant if p<0.05

Fecundity of mosquitoes that feed with AMF were lower than control. This could be due to mosquitoes imbibing serum only at the later part of the feeding process, as separation of serum and blood cells as separation about 20 minutes after blood AMF exposure[5]. As serum contains less protein than whole blood, the lower fecundity could be due to lower protein intake[13,28].

Ramos (2007) stated that calcium is an important ion in the mechanism that regulated fusion of yolk granule membrane for macro-molecule transfer between different compart-ments inside the egg. In the absence of calcium, there is no vitelline proteolysis occured. So, it can be a reason why in anticoagulant groups that chelating calcium, the vitellogenesis process can be reduced and make the production of egg decreases. Calcium-binding protein plays some role on transport mecahnisms to the oocytes during oogenesis[25].

The K_3EDTA increased the erythrocyte osmotic fragility compare to heparin and plasma in K_3EDTA had significantly higher osmolality than that in heparin. Erythrocyte fragility seems to be affected by K_3EDTA by the chelation of calcium. Na-K-Cl co transport activity increases in response to osmotic cell's shrink that induces a net uptake of solute via Na-K-Cl co transporter in a process that is regulated by a volume-sensitive protein kinase[29]. The K_3EDTA and heparin may change the levels of calcium and sodium in the serum[30].

Calcium is an element that is essential in the regulation of a great variety of biological processes. In blood plasma calcium is present at concentration of 2.2-2.6 mM (1.1-1.3 mM as free ions). Calcium ions bind to a number of coagulation protein and modulate their functions. Each factor binds 10 Ca ions at the N-terminal Gla domain and Gla-independent Ca. Calcium stabilized conformation of coagulation factor IX. The physiological level of free ions calcium (1-2 mM) is insufficient to bring full conformational change and concomitant expressions of biological activity[31].

Blood feeding triggers egg development in an autogenous mosquitoes. *Ae. Aegypti* generally produce less than 100 eggs every gonotrophic cycle[6]. Body size in mosquitoes correlated with the number of ovarioles[25]. Colless and Chellapah (1960) discovered that the quality of blood ingested from trace nearly 5 mg, and mosquitoes that ingested less than 0.5 mg failed to initiate vitellogenesis and egg maturation.

Hatchability

There was a significant differencein hatchability among trials ($p<0.05$). The hatchability of *Ae. aegypti* fed through both trials ranged from 99.49 %, 99.88 %, 99.33 % and 99.12%. There were significant differences in hatchability between K_3EDTA and others trials. Our study showed that *Ae. aegypti* fed through EDTA yielded good results (Tabel 4).

Tabel 4. Comparison of hatchability of *Ae. aegypti* fed on human control and blood with anticoagulant

Group	No.of eggs	No. of eggs that haching	Hatchability (%)
K1	8,895	8,866±11.36	99.49*
K2	3,013	3,010±9.67	99.88*
K3	7,877	7,817±9.84	99.33*
K4	4,559	4,513±11.22	99.12*

K1 : Control mosquitoes group with human direct feeding
K2 : K_3EDTA blood feeding group with AMF
K3 : Heparin blood feeding group with AMF
K4 : Sodium citrate blood feeding group with AMF
p* Kruskal-Wallis, significant if p<0.05

Pre adult development

The preadult development of *Ae. aegypti* is within a normal range as seen in Table 5.

Table 5. Comparison of pre adult developmentof *Ae. aegypti* fed on human control and blood with anticoagulant

Group	Pre adult development (days)			No. of total adult	p*
	egg-larva	larva-pupa	pupa-adult		
K1	4.34	3.63	4.03	8,816	0.000
K2	4.12	2.96	3.36	2,991	
K3	4.16	3.29	3.53	7,699	
K4	4.33	3.49	3.60	4,442	

K1 : Control mosquitoes group with human direct feeding
K2 : K_3EDTA blood feeding group with AMF
K3 : Heparin blood feeding group with AMF
K4 : Sodium citrate blood feeding group with AMF
p* Kruskal-Wallis, significant if p<0.05

Pre adult development eggs into larva in this study have range from 4.12 to 4.34 days. Eggs can hatch at range of 2-7 days[16]. K_3EDTA have brief period in pre adult development than the heparin and sodium citrate groups. It is thought to be related to the population density of eggs that hatched. Pre adult development of mosquito is more influenced by temperature, pH of water, feed, organic matter, light and predator's populations[8]. The number of eggs that hatched in the group K_3EDTA also less than that of other groups, so the development of eggs into larvae in the group K_3EDTA was faster than other groups. The control group had a total population more than any other groups so the hatch period is also longer than the other groups, although still within the range of normal period.

The mean pre adult development of larvae become pupae in this study was 2.96 to 3.63 days. Older larvae become pupae in the normal developmental stages of *Ae. aegypti* takes 2 days for male pupae and 2.5 days for females. There was a lengthening of the period of larval development into pupae[31]. It can be caused due to lack of population density uniformity pupae stage. The control group had a mean progression (3.63 days) longer than the group K3EDTA (2.96 days). This is because the population of the control group over many other groups and groups K_3EDTA population is much less than the other groups. The pre adult development pupae stage ranges 3-7 days into adulthood[31]. This study showed that the average of adult development was from 3.36 to 4.03 days, meaning still in the normal range. Vitelline is one of the substances that are important in the development process of pre adult mosquitoes[15]. Body fat is also very important in the development of the larval stage, because it is useful for storing nutrients that will be used in the development of the adult stage of mosqutoes[32]. The formation of body fat is influenced by the amount and quality of amino acids in the blood and transported into the cells by endocytosis mechanisms.

Survival rate

In average, more than 99% of *Ae. aegypti* supported by both trials can survive for 30 days throughout the generations. The mean of survival rate of *Ae.aegypti* fed on control blood and blood with anticoagulant was not significantly differ (p=0.235) (Table 6).

Table 6. Comparison of mean of survival rate of *Ae.aegypti* fed on human control and blood with anticoagulant

Group	No. of moasquitoes that blood feeding sucsses	No. of mosquitoes that can survive 30 days	Survival rate (%)	p*
K1	147±2.35	147±2.35	100	0.235
K2	66± 2.19	66±2.19	100	
K3	148± 3.14	147±2.94	99.30 (95.83-100)	
K4	92± 3.08	90±2.96	97.82 (90-100)	

K1 : Control mosquitoes group with human direct feeding
K2 : K_3EDTA blood feeding group with AMF
K3 : Heparin blood feeding group with AMF
K4 : Sodium citrate blood feeding group with AMF
p* Kruskal-Wallis, significant if p<0.05

Survivorship in vector mosquitoes is related to temperatureand available nutrition[8].

CONCLUSION

We conclude that heater can increase the blood feeding sucsses. The K_3EDTA, heparin and sodium citrate can affect the feeding rate, fecundity, hatchability, and preadult development, but do not affect survival rate. Heparin can be used for routine colonization of laboratory strain of *Aedes aegypti* with AMF assay.

SUGGESTIONS

The results of this study illustrate how variations of the human blood containing anticoagulants affect the reproductive cycle of *Ae. aegypti*. The feeding rate of mosquitoes fed on blood with anticoagulant was significantly lower than that of control direct human feeding. However, despite the reduction in weight blood meal, mosquitoes that fed on anticoagulant-added human blood produce fewer eggs, but the blood feeding success was same as control in heparin-added blood. Feeding on anticoagulant-added blood had no impact on the probability that mosquitoes would survive until 30 days after blood feeding. Lower percentage of the stages of mosquitoes in trials was caused by the less population of the mosquitoes in the paper glass rearing.

Further studies are recommended to investigate the effect of EDTA in reducing the level of calcium in plasma or serum. Nearly all routines blood feedings of *Ae. aegypti* can use heparin. Our results show that heparin rather than EDTA and sodium citrate should be used as the anticoagulant of choice in blood feeding for *Ae. aegypti* in laboratory setting.

REFERENCES

1. Departemen Kesehatan RI. *Demam Berdarah Dengue di Indonesia Tahun 1968-2009*. Buletin Jendela Epidemiologi. Vol 2, 2010.
2. Gaio AO, Gusmao DS, Santos AV, Berbert-Molinna MA, Pimenta PFP, Lemos FJA. Contribution of midgut bacteria to blood digestion and egg production in *Aedes aegypti* (diptera: culicidae). BMC *Parasites & Vectors*, 2011;4:105.
3. Zhou G, Miesfeld R. Differential utilization of blood meal amino acids in mosquitoes*J Insect Physiol*, 2009;1:1-12.
4. Ferdowsian HR, Beck N. Ethical and scientific consideration regarding animal testing and research. *PloS One*, 2011;6(9):e24059
5. Deng L, Koou SY, Png AB, Ng LC, Lam-Phua SG. A novel mosquito feeding system for routine blood-feeding of *Aedes aegypti* and *Aedes albopictus*. *Trop. Biomedicine*, 2012;29(1):169-74.
6. Takken W, Klowdwen MJ, Chambers GM. Effect of body size on host seeking and blood meal utilization in *Anopheles gambiaesen-sustricto* (Diptera:Culicidae): the disadvantages of being small. *J Med Entomol*, 1998; 35:639-45.
7. Lyski ZL, Saredy JJ, Ciano KA, Stem J, Bowers D. Blood feeding position increase seccess of recalcitrant mosquitoes. *Vector Borne Zoonot Dis*, 2011;11(8):1165-71.
8. Clements AN. *The Biology of Mosquitoes: Development, Nutrition, and Reproduction,* vol.1. New York, NY: CABI Publishing, 1992.
9. Mardihusodo SJ, Satoto TBT, Mulyaningsih B, Umniyati SR. & Ernaningsih. Bukti adanya penularan virus dengue secara transovarial pada nyamuk *Aedes aegypti* di Kota Yogyakarta. *Simposium Nasional Aspek Biologi*

Molekuler, Patogenesis, Manajemen dan Pencegahan KLB; 16 Mei 2007: Yogyakarta, 2007.

10. Wold Health Organizations. *Use of Anticoagulants in Diagnostic Laboratory Investigations and Sability of Blood, Plasma and Serum Sampels*. Genewa, 2002.
11. Solarte Y, Manzano MR, Castilo Z, James MA, Herrera S, Arevalo-Herrera M. Effects of anticoagulant on *Plasmodium vivax* oocyst development in *Anopheles albimanus* Mosquito. *Am.J.Tro.Med.Hyg*, 2007; 77(2): 242-5.
12. Patel N. Why is EDTA the anticoagulant of choice for haematology use. *Tech Talk*, 2009; 7(1).
13. Bowen RAR, Hortin GL, Csako G, Otanez OH, Remaley AT. Impact of blood collection devices on clinical chemistry assay. *Clin. Biochem*, 2009;43:4-25.
14. Katzung BG. *Farmakologi Dasar dan Klinik*. Penerbit Buku Kedokteran (EGC). Jakarta, 2010.
15. Bousema T, Dinglasan RR, Morlais I, Gouagna LC, van Warmerdam T, Awono-Ambene PH, *et al*. Mosquito feeding assay to determine the infectiousness of naturally infected *Plasmodium falciparum* gametocyte carrers. *PLoS One*, 2012;7(8): e42821.
16. Foggie T, Achee N. Standard operating procedures: rearing *Aedes aegypti* for the HITSS and box laboratory assays training manual., *Training manual*, 2009;(1):1-12.
17. Ribeiro JMC. Role of saliva in blood-feeding by arthropods. Annual Rev. of Entomol, 1987;32:463-78.
18. Galun R.Feeding stimuli and artificial feeding. *Bull World Health Organ*, 1967; 36: 590-3.
19. Davis EE, Sokolove PG. Lactid-sensitive recetors on the antennae of the mosquito, *Ae. aegypti.* J Comp Physiol, 1976;A105:43-54.
20. Ghaninia M, Larsson M, Hansson BS, Ignell R. Natural odor ligands for olfactory receptor neurons of the female mosquito *Aedes aegypti*: use of gas chomatography-linked single sensilium recording. J ExpBiol,2008;211:3020-7.
21. Chilaka N, Perkins E, Tripet F. Visual and olfactory associative learning in the malaria vector *Anopheles gambiae*sensustricto. Malaria Journal, 2012;11:27.
22. Mukabana WR, Takken W, Coe R, Knol BGJ. Host-specific cues cause differential attractiveness of Kenyan men to the African malaria vector *Anopheles gambiae*. Malaria JourNal, 2007;1:17.
23. Fernandez L & Briegel H. Reproductive physiology of *Anopheles gambiae* and *Anopheles atropavus*. J of Vec.Eco, 2004; 30; 1:11-27.
24. Chadee DD, Beier JC, Mohammed RT. Fast and slow blood feeding durations of *Aedes aegypti* mosquitoes in Trinidad. *J. of Vect.Eco*, 2002;27(2):172-7.
25. Briegel H. Metabolic relationship between female body size, reserves, and fecundity of *Ae. aegypti*. *J.Insect Physiol*, 1990; 36(3): 165-72.
26. Hogg JC, Thomson MC, Hurd H. Comparative fecundity and associated for two siblings species of the *Anopheles* occuring sympatrically in the Gambia. *Med Vet. Entomol*, 1998; 35: 639-45.
27. Bellamy RE, Bracken GK. Quantitative aspects of ovarian development in mosquitoes. *Canadian Entomol*, 1971;103:763-73.

28. Narayan S. *The preanalytic phasean important component of laboratory medicine*, 2000; 429-52.
29. Mafuvadze B, Erlwanger KH.The effect of EDTA, heparin and storage on the erythrocyte osmotic fragility, plasma osmolality and haematocrit of adult astriches (*Struthiocamelus*). Vet.Arhiv, 2007;77(5):427-34.
30. Perelman B. *Health management and veterinary procedures. In The Ostrich: Biology, Production and Health*. CABI publishing, CAB International, UK, 1999;321-46.
31. Sekiya F, Yamashita T, Atoda H, Komiyama Y, Morita T. Regulation of the tertiary structure and function of coagulation factor IX by magnesium (II) ions. J.Biol.Chem, 1995; 24(270):14325-31.
32. Emami SN, Ranford-Cartwright LC, Ferguson HM.The impact of low erythrocyte density in human blood on the fitness and energetic reserves of the African malaria vector Anopheles gambiae. *J. Malaria*, 2013;12:45.