Evaluation of Effective Dose with Protection Time of Deet by using Multi-Chamber-Blood-Feeding System against *Aedes aegypti*

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Abstract

Test on repellents were typically performed on shaven animals, such as rabbits, dogs, guinea pigs, chicks, and sheep, as alternative subject to humans. However, these techniques often raise queries concerning the ethical treatment of animals and human values or practical aspects, which sometimes misinterpret the results of repellent tests. In this study, we developed a test method and improved the evaluation of mosquito repellency time. This method was designed to reduce the risk of contamination with repellents, for evaluation of the correct effective dose and protection time. DEET (N,N-diethyl-meta-toluamide) was used as a gold standard repellent for testing the equipment for five different concentrations. The method was used to impregnate the net, size (5×5) 25cm^2 with five different concentrations and placed on the cups for testing the protection time. The protection time was recorded 5 minutes after 30 minutes of exposure. The results demonstrate that DEET with 2.5% concentration provided 3 hours of protection while the remaining concentrations 0.75%, 0.50%, 0.25% and 0.01% can protect, 120, 90, 60 and 30 min respectively. The data was analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple comparison by SPSS for Windows. The effective dose ED_{50} of DEET was (0.39%) which was calculated by Plot (log dose and % of inhibition). This method indicates that in future this innovative testing method can not only be used for repellent testing, but it can also be used for insecticide.

Keywords: *Aedes aegypti, DEET, Net Impregnation, Membrane Feeding, Multi-chamber-blood-Feeding system*

1. Introduction

Since 1919 several sound laboratory cage tests were conducted by using several repellents against *Aedes aegypti* (Bacot and Talbot, 2009). After that, a variety of tests were attempted and was being published. Later Christophers in 1947 noted that standardized test procedures and assessment criteria was needed, but there was still a great deal of discussion about a true consent regarding the best repellent test method. Mosquito repellents applied on the human skin are among the oldest and most common methods for protecting people and in many contingencies, it was the only way to avoid mosquito bites (Fradin & Day, 2002). But some synthetic repellents left toxic reactions on human skin after application such as DEET (N,N-diethyl-meta-toluamide) when applied on the skin its toxicity reaction on human skin may vary from mild to severe (Edwards & Johnson, 1987). Later in 1987, the National Research Council (1987) recommended the use of animals instead of a human subject for mosquito repellent testing. (National research center 1987). This recommendation was based on the safety of human subject concern who may be exposed to test materials for which toxicity information was inadequate or incomplete. However, repellents were typically performed on shaven animals such as Rabbits, dogs (Hill, Robinson, McVEY, Akers, & Reifenrath, 1979), guinea pigs (Kasman S. et al., 1953), chicks, (Watanabe, Takada, Matsuo, & Nishimura, 1995) and sheep, as alternative subjects to humans (Nicolaides, Fu, & Rice, 1968). However, these techniques often raised queries concerning the ethical treatment of animals and human's ethics or practical aspects, which sometimes distorted the results of repellents tests.

However, some studies had utilized membrane blood-feeding for mosquitoes, used in repellency trials (Rutledge, Moussa, & Belletti, 1976), although such data obtained using this technique should be considered as initial indicators for final confirmation in tests involving human subjects (Parks, & Bryan, 2001). Membrane feeders were used when rearing mosquitoes at large scale or when there is a concern about animal welfare or where it is not feasible to use human volunteers for ethical or practical reasons (Gonzales &

Hansen, 2016). To keep all these challenges in mind, this study was designed to develop a method for testing which is an alternative to human arm in cage method or other studies that used animals as a model for testing. This method is easy to conduct and not only for repellent testing but can also be used for insecticide testing.

2. Objective

1. To develop a method for repellent testing without any exposure risk to human volunteer.
2. To evaluate the effective dose (ED_{50}) and protection time of DEET by using Multi-Chamber-Blood-Feeding system against *Aedes aegypti*.

3. Materials and Methods

3.1 Ethics statement: Mosquito testing for DEET testing was approved by the Mahidol University Animal Care and Use Committee (FTM-ACUC) (Approval U1-01859-2558).

3.2 Test insects: The present repellent testing was conducted on Laboratory strain *Aedes aegypti* (Bora Bora). The colony was maintained in the laboratory condition 25±2°C, 60±10% RH and 12L:12D photoperiod at the Laboratory of Tropical Disease Research Center, Kanchanaburi, Faculty of Tropical Medicine, Thailand. Rearing conditions and procedures were as stated by Rutledge, Moussa, Lowe, and Sofield (1978). Nulliparous females 5-7 days old were used for repellent testing and starved for at least 8 hours before starting the experiment.

3.3 Test Repellent: DEET was used as a repellency testing which is known as gold standard for comparison of other material testing. There were five different concentrations of DEET (0.01%, 0.25%, 0.50%, 0.75% and 2.5%), were prepared by mixing with the absolute ethanol in amber glass bottles and shaken well.

3.4 Net Impregnation: The impregnated nets were prepared, size (5×5=25cm^2) diameter. The volume of each concentration was fixed 21µl dropped on the center of net, the nets were impregnated with five different concentrations mentioned above. After impregnation, the nets were allowed to shade dry for 5 minutes, and then packed into foil without any risk of contamination at -4°C and was used at the time of experiment. For positive control nets were impregnated with absolute ethanol.

3.5 Chamber Composition: The idea for repellent testing was designed by combining blood supplied system together with the closed system of bioassay chamber Figure 1. This method allowed multiple testing and comparison for various types of materials, dosages or replication of the repellents. The rectangular chamber was dimensioned (35 x 35 x 35 cm^3) and made with metal tube of 1cm diameter. Each chamber was fixed together with the metal tubes. The repellency test of the DEET was carried out by preparation of 4 components; 1) the multi-chamber, 2) the impregnated net, 3) the tested cups, 4) the tested mosquitoes. There were total 6 chambers for testing, one was a control and the rest of the chambers were used for five different concentration of DEET. A side wall of each chambers was still opened to facilitate installation of the blood supplied system and the tested cups. This side wall was closed with plastic when all installation and the releasing mosquito into the tested cups are completed.

3.6 Repellency bioassay: Before testing with the tested repellent, the equipment was tested with positive control (no-net) to check the mosquito feeding status, light temperature, relative humidity etc. after three times replication, then the equipment was tested with positive control (absolute ethanol impregnated net) when every chamber was tested 6 times, the chamber passing criteria was 3/5 mosquito feeding all the chambers. If less than 3 it means the chamber is contaminated or other factors which affect the outcome of the result. After passing, the experiment was started with test repellent concentrations. The repellency bioassay was started when all the cups were placed on their specified chambers after that the impregnated net were placed on the desired cups and keep the feeder on it to allow for feeding for 5 minutes of exposure. Starting time for releasing the mosquito into each tested chamber was lagged 10 seconds to facilitate the exact duration of monitoring in each chamber even using only one staff. Applying the number of mosquitoes from the similar idea from WHO cone test guideline (WHO, 2013), 5 female mosquitoes were released in each tested cup with the help of aspirator for experiment. During 5 minutes of exposure the number of repel and the blood fed

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mosquitoes were recorded. If there is no feeding in any chamber, the net was again packed into the foil and keep at -4°C. After 30 minutes interval, the same impregnated net was again tested with fresh batch of adults to ensure that failure to bite was due to repellency potential of DEET and not because of the mosquitoes being pre-disposed to get a blood meal. Total fifteen replications were done by each tested concentration to ensure the promising results.

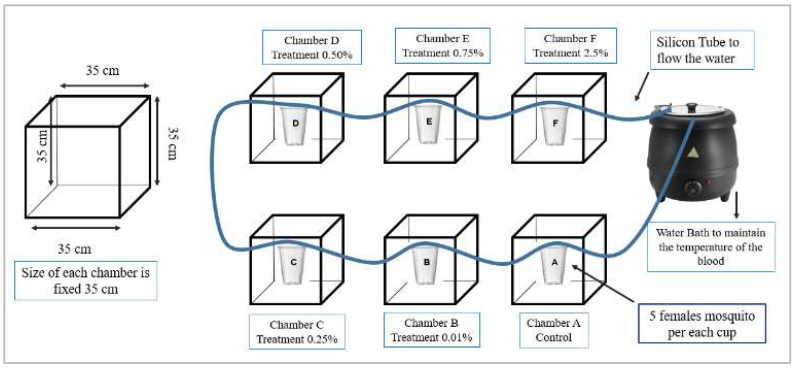

Figure 1. Multi-Chamber-Blood-Feeding System for testing of repellent

3.7 Statistical analysis: Protection time was recorded as the time elapsed between the 1st time round of impregnated net and the time of confirmed bite. The mean protection time was used to compare the five different concentrations of DEET with absolute ethanol as a control. Differences in significance were analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple comparison by SPSS for Windows.

For comparison, a percentage of mosquito biting was calculated for each test using the following formula (Amer, & Mehlhorn, 2006; Phasomkusolsil, & Soonwera, 2010).

% Biting = B × 100/total number of tested Mosquito

4 Results and Discussion

Relative repellency (mean protection time) and biting percent under laboratory conditions provided by DEET with five different concentrations against *Aedes aegypti* are summarized in Table 1. Control mean biting rate (*BR*) was significantly higher than the treatment chambers as shown in Figure 2. Mean BR on control chamber ranged from 78.8 to 75.89%. while in treatment chamber *BR* ranged from 25.33 to 20%. Absolute ethanol was used as a positive control.

Table 1. Data is expressed as Mean+SD. Mean in each column against *Aedes aegypti* followed by different superscript letters are significantly different by (P<0.05) by one-way ANOVA with Duncan's Multiple Range test (DMRT).

Dose%	Replication	Mean Protection time (min)	Relative (%) of Mean protection time	Biting percentage %		ED_{50}
				Control Chamber	Treatment Chamber	
0.01%	15	44 ± 15.49^{a}	25.28	75.89 ± 0.60^{a}	21.33 ± 0.60^{a}	
0.25%	15	66 ± 12.42^{b}	37.93	71.60 ± 0.58^{a}	28.00 ± 0.51^{a}	
0.50%	15	98 ± 13.73^{c}	56.32	71.33 ± 0.38^{a}	20.00 ± 00^{a}	0.39%
0.75%	15	122 ± 23.96^{d}	70.11	76.80 ± 0.71^{a}	25.33 ± 0.46^{a}	
2.5%	15	174 ± 12.42^{e}	100	78.80 ± 0.70^{a}	24.00 ± 0.41^{a}	

Relative % of mean protection time = Mean time ×100/ Highest mean time

$^{a, b, c, d, e,}$ these superscript letters showing significant difference by (P<0.05)

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There were significant differences in repellency between different doses of DEET against *Aedes aegypti* by ($P<0.05$). The data showed that chemical concentration correlated positively with repellency. The effective dose was analyzed by converting the dose% into log dose and mean protection time converted into percent of mean protection time, after that the data was showed on plot to get the ED_{50} of the tested concentrations, which was ($ED_{50} = 0.39\%$) Figure 3. (Yap, Jahangir, & Zairi, 2000) showed that 10% DEET can protect about 6 h against the bite of *Ae. aegypti.* Another study reported that when DEET applied at 6.65% and 20% its mean protection time is 110 and 230 minutes respectively (Koren, Matsui, & Bailey, 2003). It was shown that 20% DEET gave 95% protection for only 2 hrs. against all mosquito species (Frances, Cooper, Poopat, Sweeny, 1999; Frances, Mackenzie, Klun, Debboun, 2009). However, in our study 2.5% DEET can protect 3 hrs. the other four concentrations 0.75%, 0.50%, 0.25% and 0.01% with fixed volume and provided an average 120, 90, 60 and 45 minutes respectively against *Aedes aegypti*. The protection provided by DEET is proportional to the logarithm of the dose, higher dose of DEET provide long lasting protection, but the duration of action tends to plateau at a concentration of about 50% (Buescher, Rutledge, Wirtz, & Nelson, 1983).

The protection time was observed as long protection as compared to previous studies. Early researchers used human volunteers for repellent testing (Fradin & Day, 2002) which is the oldest protocol. There were many factors which affected the outcome of the repellent bioassay when human used as a subject, including absorption and penetration of repellent on skin, evaporation, abrasion (contact with clothing), washing or rinsing of treated surfaces. Perspiration also result in repellent loss and as a result these factors may misconstrue the results of repellents (Gabel, Spencer, & Akers, 1976; Rueda, Rutledge, Gupta, 1998; Xue, Barnard, & Schreck, 1995, Barnard, Posy, Smith, & Schreck, 1998). But in our study, we designed to prevent the repellent loss by using net impregnation techniques and testing with a closed chamber which we pronounce MCBF system. As in this study, impregnated net was used instead of repellent-treated fabrics or human/animal skin, higher protection time was observed. As seen in repellent testing with fabric retention of repellent for a longer period, no skin absorption loss, no effect of sweating on the active compounds, slower evaporation rate and better adherence could be pointed out as some of the benefits for using impregnated net. Impregnation of bed nets or garments with DEET can prolong its persistence (Curtis, Lines, Ijumba, Callaghan, Hill, & Karimzad, 1987; Gouck, & Moussa, 1969). Interval time was fixed for 30 minutes for exposure of impregnated net to every new batch of mosquito for repellency testing, because continuous exposure may cause fatigue to mosquitoes or could induce prolonged blockage of their antennal chemoreceptors (Fradin, & Day, 2002).

If we compare our study with the previous research studies there is a great difference in terms of protection time, and biting rate etc. There were unlimited factors which may have effects on the outcome of tested chemicals including environmental conditions, mosquito species, test subjects, chemicals formulas, application techniques and design of the study (Fradin & Day, 2002; Barnard D. R. 2003; Golenda, Solberg, Burge, Gambel, & Wirtz, 1999; Schreck, 1977). Although laboratory testing provides a general product effectiveness indicator, various factors like reduced evaporation of the product and lotion breakdowns have a substantial effect on their application outside of the field. Field tests however continue to be the basis for the determination of the true effectiveness of an insect repellent and continue to be the only requirements for EPA approval for such products (Environmental Protection Agency, U.S. 1999; Environmental Protection Agency, U.S. 2000).

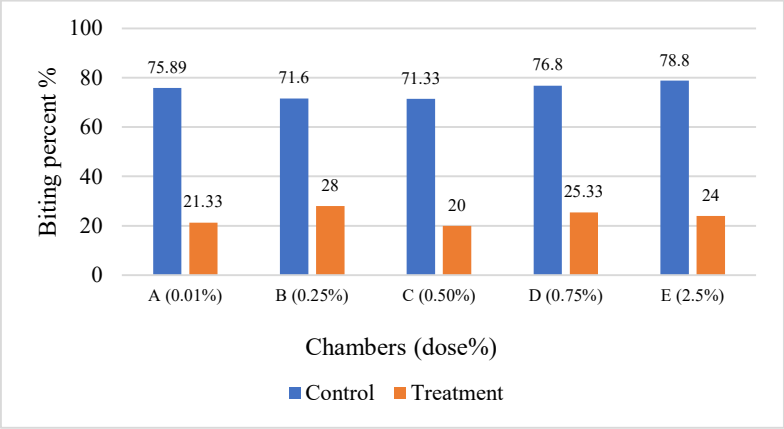

Figure 2. Comparison of Biting percent among the control and treated groups.

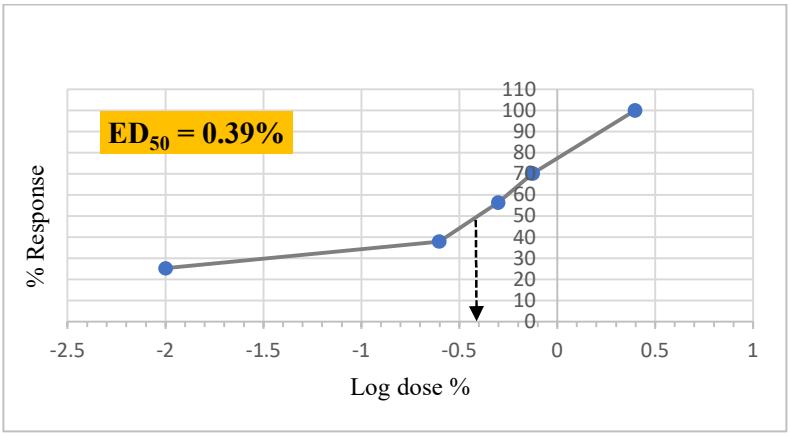

Figure 3. Log dose response curve and LD_{50} of DEET against *Ae. aegypti*

4. Conclusion

Mosquito repellency property on the net could be achieved by the application of many repellents/chemicals. However, most of these chemicals showed some detrimental side effects on humans and the environment when overdose application. Therefore, this technique is best for the testing of repellents as well as insecticide also. This method is easy to conduct, one person can handle all the concentrations at a time. There is no risk of repellent loss, contamination, or evaporation, etc. and this method can be used where humans or animals could not be used for any practical or ethical concern. As this method has many advantages, on the other hand, it also has some drawbacks. We suggested that when testing with a high dose of any botanical oil or repellent, this can affect the parafilm and there is a risk of blood leaking out. So, it is preferable to use animal skin such as, pig intestine, cow intestine, or chicken skin, which also provide the real environment for the mosquito to attract for feeding toward the feeder.

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6. References

Amer, A., Mehlhorn, H. (2006). Repellency effect of forty-one essential oils against Aedes, Anopheles and Culex mosquitoes. *Parasitology Research, 99,* 478-490.

Bacot, A., Talbot, P. G. (2009). The Comparative Effectiveness of Certain Culicifuges under Laboratory Conditions. *Parasitology, 11*(2), 221-236.

Barnard, D. R., Posey, K. H., Smith, D., and Schreck, C. E. (1998). Mosquito density, biting rate and cage size effects on repellent tests. *Medical Veterinary Entomology, 12,* 39-45.

Buescher, M. D., Rutledge, L. C., Wirtz, R. A., & Nelson, J. H. (1983). The dose-persistence relationship of DEET against *Aedes aegypti*. *Mosquito news*, *43*(3), 364-366.

Cristophers, R. (1947). Mosquito repellents. *Journal of Hygiene, 45,* 176-231.

Curtis, C. F., Lines, J. D., Ijumba, J., Callaghan, A., Hill, N., Karimzad, M.A. (1987). The relative efficacy of repellents against mosquito vectors of disease. *Medical Veterinary Entomology. 1,* 109-119.

Edwards, D. L., & Johnson C. E. (1987). Insect-repellent-induced toxic encephalopathy in a child. *Clinical Pharmacy, 6*(6), 496-498.

Environmental Protection Agency, U.S. (1999). Biopesticide technic al document II 3509: 3 - [N - butyl-N - acetyl-amino-propionic acid, ethyl ester. Washington, DC. form http ://www.epa. gov/pesticides/biopesticides/ ingredients/factsheets/factsheell 13509.

Environmental Protection Agency, U.S. (2000). Report of the FIFRA scientific advisory panel meeting April 5-7, Arlington, VA. Washington, DC. From http: //www.epa. gov/oscpmont/sap/2000/ a pril/ free porta pill.

Fradin, M. S., & Day, J. F., (2002). Comparative efficacy of insect repellents against mosquito bites. *New England Journal Medicine. 347*(1), 13-8.

Frances, S. P., Cooper, R. D., Popat, S., Sweeny, A. W. (1999). Field evaluation of the repellents, DEET, CLC4, and AI3-37220 against Anopheles in Lae, Papua New Guinea. *Journal of the American Mosquito Control Association, 15,* 339–41.

Frances, S. P., Mackenzie, D. O., Klun, J.A., Debboun, M. (2009). Laboratory and field evaluation of SS220 and DEET against mosquitoes in Queensland, Australia. *Journal of the American Mosquito Control Association*, *25,* 174–5.

Gabel, M. L., Spencer, T. S., and Akers. W. A. (1976). Evaporation rates and protection times of mosquito repellents. *Mosquito News, 36,* 141-146.

Golenda, C. F., Solberg, V. B., Burge, R., Gambel, J. M., & Wirtz, R. A. (1999). Gender-related efficacy difference to an extended duration formulation of topical N, N-diethyl-m-toluamide (DEET). *The American journal of tropical medicine and hygiene*, *60*(4), 654-657.

Gonzales, K. K., & Hansen, I. A. (2016). Artificial diets for mosquitoes. *International journal of environmental research and public health*, *13*(12), 1267.

Gouck, H. K., Moussa, M. A., (1969). Field tests with bed nets treated with repellents to prevent mosquito bites. *Mosquito News 29,* 263.

Hill, J. A., Robinson, P. B., McVEY, D. L., Akers, W. A., & Reifenrath, W. G. (1979). Evaluation of mosquito repellents on the hairless dog. *Mosquito News*, *39*(2), 307-310.

Kasman, S., Roadhouse, L. A. O., and Wright, G. F. (1953). Studies in testing insect repellents, *Mosq News, 13,* 116.

Koren, G., Matsui, D., & Bailey, B., (2003). DEET-based insect repellents: safety implications for children and pregnant and lactating women. *Canadian Medical Association, 169* (3), 209-212.

National Research Council. (1987). Toxicity of candidate arthropod repellents: Appraisal of the Armed Forces topical hazard evaluation program. *Washington, DC: National Academy Press.*

Parks, W., & Bryan, J. (2001). Gender, mosquitos and malaria: implications for community development programs in Laputta, Myanmar. *The Southeast Asian journal of tropical medicine and public health*, *32*(3), 588-594.

Phasomkusolsil, S., Soonwera, M. (2010). Insect repellent activity of medicinal plant oils against Aedes aegypti (Linn.), Anopheles minimus (Theobald) and Culex quinquefasciatus (Say.) based on protection time and biting rate. *Southeast Asian Journal of Tropical Medicine Public Health, 41*(4), 831-840.

Rueda, L. M., Rutledge, L. C., and Gupta, R. K. (1998). Effects of skin abrasion on the efficacy of the repellent deet against *Aedes aegypti*. *Journal of the American Mosquito Control Association*,*14*, 178-182.

Rutledge, L. C., Moussa, M. A., & Belletti, C. J. (1976). An in vitro blood-feeding system for quantitative testing of mosquito repellents. *Mosq. News*, *36*(3), 283-293.

Rutledge, L. C., Moussa, M. A., Lowe, C. A., & Sofield, R. K. (1978). Comparative sensitivity of mosquito species and strains to the repellent diethyl toluamide. *Journal of medical entomology*, *14*(5), 536-541.

Schreck, C. E. (1977). Techniques for the evaluation of insect repellents: a critical review. *Annual review of entomology*, *22*(1), 101-119.

Watanabe, K., Takada, Y., Matsuo, N., & Nishimura, H. (1995). Rotundial, a new natural mosquito repellent from the leaves of Vitex rotundifolia. *Bioscience, biotechnology, and biochemistry*, *59*(10), 1979-1980.

World Health Organization (WHO). (2013). Guidelines for laboratory and field-testing of long-lasting insecticidal nets. Switzerland: (In Press).

Xue, R. D., Barnard, D. R., and Schreck, C. E. (1995). Influence of body size and age of *Aedes albopictus* on human host attack rates and the repellency of deet. *Journal of the American Mosquito Control Association, 11,* 50-53.

Yap, H. H., Jahangir, K., & Zairi, J. (2000). Field efficacy of four insect repellent products against vector mosquitoes in a tropical environment. *Journal of the American Mosquito Control Association*, *16*(3), 241-244.