Guideline to Efficacy Evaluation of Mosquito Repellent

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MINISTRY OF FOOD AND DRUG SAFETY

National Institute
of Food and Drug Safety Evaluation

Revision of Guideline to Efficacy Evaluation of Mosquito Repellent

1. Reason of Revision

To provide safe quasi-drugs to nation and support development of products in industry by adding efficacy evaluation method of adhesive* mosquito repellent

* Adhesive: A type of band or patch containing mosquito repellent which is worn in wrist or ankle

2. Main Contents

A. Addition of efficacy evaluation method of adhesive mosquito repellent

❍ 2. Paragraph of <Overview> for calculation of complete protection time (CPT) in repellent efficacy test methods in laboratory

However, adhesive mosquito repellent should be tested after attaching it to the test area (arm parts from wrist to elbow) in accordance with dose and administration of the product.

❍ 2. Paragraph of <Test Method> for calculation of complete protection time (CPT) in repellent efficacy test methods in laboratory

Adhesive mosquito repellent should be tested after attaching it to the test area in accordance with dose and administration of the product.

❍ 3. Paragraph of <Test Method> for efficacy and persistency of the product in outdoor repellent efficacy test

Adhesive mosquito repellent should be tested after attaching it to the test area (wrist or ankle) in accordance with dose and administration of the product.

This guideline describes the view of the MFDS on the efficacy evaluation method of mosquito repellent, quasi-drug, and it does not have legal effect publicly

※ Guideline refers to public description of the view of MFDS on certain matters (Regulation on Management of guidelines, etc. of MFDS (MFDS established rules))

※ If you have opinions or questions on this guideline, contact Cosmetics Evaluation Division, Biopharmaceuticals and Herbal Medicine Evaluation Department in National Institute of Food and Drug Safety Evaluation.

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History of Enactment and Revision

Enactment and Revision No.	Date of Approval	Main Contents
B1-2012-3-005	Nov. 27, 2012	Enactment of guideline to efficacy evaluation method of mosquito repellent
B1-2015-3-006	Aug. 17, 2015	Revision of guideline to efficacy evaluation method of mosquito repellent

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1. Introduction

The types of hygienic harmful insects are various including mosquitos, flies, cockroach, mite, etc., but mosquito, particularly, can be mediate fatal diseases such as encephalitis, malaria, etc. Thus, more effective mosquito repellent development is being required. Mosquito repellent does not have effect of killing mosquito, but it contains the substances mosquito dislike. If it is applied or sprayed in the skin or clothes of humans, this product prevents mosquito to suck the blood. The repellent is classified as quasi-drug, our department approves and manages it.

To evaluate the accurate repellent efficacy of the mosquito repellent to human body, a variety of factors should be considered to obtain more accurate and reproducible results. Therefore, this guideline attempts to provide standardized test method which can be helpful for efficacy test and evaluation of mosquito repellent.

This guideline provides a guide to evaluate the efficacy of mosquito repellent through laboratory and outdoor tests of the active ingredients used in the mosquito repellent.

This guideline does not handle with the safety of the mosquito repellent. To apply the test materials to human skin, preliminary safety evaluation on persons should be preceded. Accordingly, it is necessary to closely observe the side effects or undesirable features related to application and use of mosquito repellent in laboratory and outdoor tests.

The laboratory insects used in the biological test may be changed by rearing environment and a breeder. The test needs the instruction of experienced operator due to the characteristics of the biological test of the repellent. In addition, since the test of repellent is conducted in humans, the sufficient safety of subjects and confidence of the test result should be secured.

2. Laboratory Repellent Efficacy Test Method

<Overview>

The purpose of this test is to measure the effective dose (ED) of the mosquito repellent and complete protection time (CPT) after applying the repellent to the skin. In this test, 50% and 99.9% of ED (ED_{50}, $ED_{99.9}$) of the repellent preventing mosquito to land and/or suck blood and CPT from the initial application of the repellent to landing and/or bloodsucking should be measured in detail.

<Efficacy Judgment>

The CPT of the test material should be 2 hours and longer.

2.1. General Statements

<Positive Control>

As the positive control group of the mosquito repellent, 20% N,N-diethyl-3-methylbenzamide(Deet) ethanol solution.[1)]

<Selection of Testing Mosquito>

To test efficacy of mosquito repellent, Aedes(*Aedes aegypti*, A*edes albopictus*) species, Culex(*Culex Pipiens* Pallens) species and Anopheles (*An. stephensi*, *An. gambiae or An. albimanus*) which like humans will be used.

<Preparation of Testing Mosquito>

The followings should be considered in the preparation of test mosquito.

- Test species, system and age of the week of mosquitos used in the repellent test should be recorded.

1) 20% N,N-diethyl-3-methylbenzamide(Deet) ethanol solution: Weigh about 2 g of N,N-diethyl-3-methylbenzamide, melt it in ethanol, and make 10mL.

- The mosquitos used in the test should be the ones with aged 5 ~ 7 days after ecdysis from pupa.
- Here, the mosquitos should be bred in an insectarium in which male and female insects exist together so that mating can occur.
- Use female mosquitos which actively find host to suck blood by selecting them with an appropriate instrument such as an aspirator.
- Until the testing mosquitos are used, sugar solution should be supplied instead of blood, and fast them for 12 hours and more before the beginning of the test.

<Breeding Testing Mosquito and Test Condition>

To increase the confidence and reproducibility of the test results, standardized mosquito breeding method and test conditions are required. The mosquitos should be bred at temperature 27±2℃ and relative humidity 80±10% and over, maintaining light cycle of 12 hours. The test should be conducted in an independent, separate space from breeding area. However, breeding conditions may be changed depending on species of mosquito.

<Mosquito Insectarium>

As the mosquito insectarium, metal frame in which contamination removal is easy should be used. The width, length and height of the insectarium should be 30 ~ 40 cm per surface. In the upper and lower surfaces of the insectarium, hard materials should be used. The left and right sides should be transparent acrylic panel should be used, the back side should be screen or net, and the front should be fiber material sleeve which provides easy access.

Figure 1. Insectarium in Universiti Sains Malaysia Vector Control Research Unit

<Subject Selection and Management>

The repellent test conducted in persons is the method to derive similar results as the actual use condition by using the end consumers of the repellent. If laboratory animals or artificial membrane is used, inappropriate results may occur from the situation where the repellent is used in human skin. As subjects, healthy adult volunteers who have no hypersensitivity or weak reaction to mosquito bite should be selected. Before the test, the approval of Institutional Review Board (IRB) is required, and overall progress should be in accordance with Good Clinical Practice (GCP). It is desirable to have the same number of male and female subjects.

The skin of the subjects being tested should be washed with an odorless soap and rinsed with water. After that, the skin should be rinsed with 70% ethanol or isopropanol and dried with a towel. To minimize various factors that may change the aggression of mosquito against people and factors that may affect the analysis results, the subjects should not use perfume and other repellents 12 hours before the test and during the test. If possible, the subjects should be non-smokers, and even smokers should not smoke cigarettes 12 hours before the test and during the test.

2.2. Effective Dose Test (Active Ingredient)

<Overview>

This is the test to identify the range of ED by using ethanol or adequate diluting solvent to produce repellent diluent. Use the dose with 10 ~ 90% of repellent efficacy, and select 2 ~ 3 doses less than 50% of repellent efficacy, and 2 ~ 3 doses with 50% and more efficacy and use them.

<Test Method>

Apply 1 mL of ethanol or diluted solvent used in dilution of repellent to about 600 cm^2(see Attachment 1) in forearm between wrist and elbow and dry it (dry about 1 minute for ethanol) and make the forearm as control group. Put the arm in which diluted solvent is applied in the test insectarium containing 50 ~ 100 female mosquitos, and record the number of mosquitos landing or bloodsucking on the skin for 30 seconds (at this time, were gloves in a hand with materials that prevent mosquitos to suck blood). During the test, the subjects should not move their forearms. As the experiment is progressed, the ratio of bloodsucking mosquitos should be landing for 30 seconds or bloodsucking 10 times.

After removing the forearm used as the control, apply 1 mL of the repellent diluent with the lowest concentration and then dry it. Put this forearm in the test insectarium for 30 seconds again, and record the landing or bloodsucking of mosquitos. Repeat this process while increasing the dose of repellent gradually. Conduct the experiment continuously without delay, and the dose of repellent in the consecutive experiments should be calculated as the sum of the applied concentrations (Table 1).

Each subject should increase dose in the tested forearm, and use at least 5 and more doses. Each test should use the same mosquitos for the same subject, and be completed in 1 day. As repeated test, repeat this process using mosquitos in other batches for several days. It is recommended to conduct repeated tests at least 3 times per subject, and for statistical analysis, a sufficient number of subjects are required.

Table 1. Examples of Consecutive Doses Applied to Reach Accumulated Dose

Application Order	Concentration of repellent (mg/mL) in application of 1 mL	Accumulated dose of repellent (mg/600 cm^2 area)
Left arm control	Preliminary treatment with a diluting solvent[s]	–
Left arm dose 1	1	1
Left arm dose 2	1	2
Left arm dose 3	2	4
Left arm dose 4	4	8
Left arm dose 5	8	16
Right arm control	Preliminary process with only diluting solvent[s]	–

[a] Ethanol or diluted solvent for repellent

<Test Result>

If it is difficult to count the number of mosquitos landing on the skin or bloodsucking accurately because the ratio of landing or bloodsucking is too high, calculate the ratio of average landing or bloodsucking of the mosquitos in the experiment 3 times for 5 seconds, and then multiply by 2 to calculate the number of landing and bloodsucking to occur for 30 seconds. The ratio of landing or bloodsucking of mosquitos in the forearms of subjects is less than 10 mosquitos (times) in 30 seconds, do not continue the experiment.

This process should be applied to the entire test in common, and well-trained subjects should record the number of landing and/or bloodsucking of mosquitos. After completion of the dose reaction test, apply 1 mL ethanol (diluting solvent) in another forearm and dry it. Then, put the forearm in the testing insectarium for 30 seconds as same as the beginning of the test, and then identify whether the number of landing and/or bloodsucking mosquitos reaches 10 (times) or more. If the ratio is not more than 10 mosquitos (times) for 30 seconds, conduct the test again.

<Calculation and Analysis>

Repellent effect (p) should be expressed as the number of landing or bloodsucking mosquitos (T) on the forearm treated with the repellent compared to the number of landing or bloodsucking mosquitos (C) on the control forearm of each subject.

$$p = 1 - (T / C) = (C - T) / C \qquad ①$$

Here, C refers to the mean of the number of landing and/or bloodsucking mosquitos on 2 forearms in which repellent is not treated (forearms applied by diluting solvent before repellent treatment and another forearm at the end of test). ED_{50}, $ED_{99.9}$ and their confidence intervals should be analyzed using probit-plan regression analysis.[2)]

2) http://probit-anaysis-software.fyxm.net

2.3. Calculation of CPT of Repellent Efficacy (Active Ingredient and Agent)

<Overview>

The CPT can be measured with 1 mL of sample of repellent of $ED_{99.9}$ by the test method in 2.1 for 20% N,N-diethyl-3-methylbenzamide(Deet) ethanol solution 1 mL, or by applying the samples of repellent in different arms and comparing them for 20% N,N-diethyl-3-methylbenzamide(Deet) ethanol solution 1 mL. In these 2 cases, the drug should be treated in the area of 600 cm^2 in the skin from the wrist to elbow (Attachment 1). However, regarding adhesive[3] repellent, attach the product in the test area (arms from the wrist to elbow) according to the dose and administration of the product.

<Test Method>

Generally, use 2 testing insectariums containing 200 ~ 250 female mosquitos that haven't sucked the blood. Among them, 1 testing insectarium is for the test of repellent sample, and the other one is for positive control[20% N,N-diethyl-3-methylbenzamide(Deet) ethanol solution]. Here, the subject should wear gloves with material in which mosquitos cannot suck the blood to protect hands. The subject should not move his or her hand during the test. When putting the arms treated with diluting solvent for 30 seconds, the number of landing or bloodsucking of mosquitos should be not less than 10 mosquitos (times).

Apply the sample of 1 mL repellent prepared by ethanol or diluting solvent before the test in one arm, and apply 20% N,N-diethyl-3-methylbenzamide(Deet) ethanol solution 1 mL in the other arm. In terms of adhesive mosquito repellent, attach it according to the dose and administration and test it. After 30 minutes, put the arm treated with the repellent sample in the applicable testing insectarium for 3 minutes, and measure the landing and/or bloodsucking activities of mosquitos. 20% N,N-diethyl-3-methylbenzamide(Deet) Evaluate the arms treated with ethanol solution in the same way. Repeat the test at interval of 30 minutes or 60 minutes.

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3) Adhesive: A type of band or patch containing repellent which is worn in wrist or ankle

<Result Judgment and Analysis>

When 1 landing and/or bloodsucking of mosquitos is made in 3 minutes, the test on the applicable dose of repellent may be completed. CPT should be calculated by the minutes between repellent application time and the first landing and/or bloodsucking of mosquito. The test for drug substances of most repellents should be completed within 8 hours. When an arm treated with anything is put in the 2 testing insectariums prepared for the test, if landing and/or bloodsucking of mosquitos are is 10 mosquitos (times) for 30 seconds, test it again.

The number of subjects participating in this test should be sufficient to do statistical analysis. The average CPT and confidence interval are calculated by Kaplan-Meier survival function (Attachment 2).

3. Outdoor Repellent Efficacy Test Method

<Overview>

To evaluate the optimal application dose, persistency and efficacy of the active ingredient of repellent, the result of laboratory test which evaluates repellent and CPT is expanded to one or more mosquito or harmful insect species in other ecological, geographical environment. It is recommended to conduct at least 2 outdoor tests. Conduct 1 test each in other ecological and/or geographical environments appropriate for the target mosquito species to which humans can be exposed. Evaluate the mosquitos landing or bloodsucking in one or more parts in arms and legs of the subjects (from knee to ankle and from elbow to wrist) in a way that the subject collect the mosquito themselves. Regarding the efficacy of repellent and durability, it is recommend to use proportional endpoint determination. This method facilitates the evaluation of ED, efficacy time in the dose range, half-life of repellent, and CPT at the same time.

<Judgment of Efficacy>

The CPT of the test substance should be 2 hours and longer.

<Subject Protection>

- The subjects should not have been exposed to specific infectious risk at the same environment as the test being conducted.
- If appropriate and applicable, the subjects should be protected by chemical prevention or vaccination.
- If possible, the test should be conducted in a place with a lot of the target mosquito species but without no disease infections.
- Before the test, approval of the Institutional Review Board (IRB) is required, and overall progresses in the test should be conducted in accordance with the Good Clinical Practice (GCP).

3.1 Efficacy and Persistency of Active Ingredient

<Overview>

This test is to measure repellent power and continuous time of the repellent sample by applying effective dose (ED) and complete protection time (CPT) from the laboratory repellent efficacy test to outdoor environment. It is recommended to evaluate 4 concentrations of the candidate repellent.

<Test Preparation>

To minimize the difference of the number of landing or bloodsucking of mosquitos by place, a preliminary survey to evaluate the adequacy of the testing place by collecting mosquitos landing in humans is required before this test. With the preliminary survey, a place with uniform mosquito population with adequate bloodsucking rates can be selected.

<Test Method>

A trained operator should collect the mosquitos landed on the exposed arms and legs using a collecting tube or aspirator (blow type or mechanical type) before the mosquitos begin to suck the blood. Transfer the mosquitos absorbed or collected to a marked cup, and replace the marked cup every 30 minutes. The test should include the same number of male and female subjects, if possible.

The collection of mosquito should be conducted in the time in which the target mosquito species are active according to habit of the mosquito. If the target mosquito species has short time of activity for bloodsucking, conduct the test, considering this time.

The subject must not use odorous products and smoke cigarettes 12 hours before or during the test. The test should be conducted in non-smokers, if possible.

Measure the area of skin in arms and legs of each subject to be used in the test (Attachment 1), and determine the amount to treat in the applicable area at a

certain concentration. To continue the efficacy during the test period, dilute the active ingredients with ethanol (or diluting solvent) to make enough amount. Before the first collection begins, apply an adequate amount of test repellent in arms or legs of the subjects evenly using a pipette, and then dry them. To provide that the subject should not recognize the characteristics of the test substance, conduct the blind test.

The subjects in the negative control should participate in the test after applying an adequate amount of ethanol or diluting solvent to their skin in the same way as the treatment of repellent. Positive control[20% N,N-diethyl-3-methylbenzamide (Deet) ethanol solution] can also be used.

In each collection place, the subjects should be located at distance of 20 m each other. 4 concentration ranges are recommended for each repellent, and the dose of repellent may be similar to the laboratory test (see 2.1). When the exposure period is completed, select the dose securing the range with large repelling ratio. Generally, the dose representing 99.9% repelling effect in laboratory test is used to the reference to determine the dose of outdoor test.

As the study design, a complete randomization should be used. The number of subjects and collection place is the same as the number added by the number of doses to be tested and negative control. If positive control [20% N,N-diethyl-3-methylbenzamide(Deet) ethanol solution] is used, additional collection places and subjects may be required. One (1 day) test is composed of all subjects going around to all collection places randomly at interval of 1 hour (Table 2). Each dose, negative and/or positive control subjects should be tested for an adequate period at least 2 days and more. The repeated test should be conducted randomly with the similar method.

In the collection place, the mosquito collection for the subjects treated with repellent and negative and/or positive control should be adequately selected according to the target mosquito species. For example, if subjects are bitten seriously, observe them for 1 minute each in 15 minutes from the application of the repellent (that is, 15 ~ 16 minutes), through 15, 30, and 45 minutes in 1 hour of observation period (0 ~ 1 hour) for 1 hour of the first measurement time.

To measure the accurate number of mosquito landing o bloodsucking and

distinguish the species, collect them with an aspirator to record the composition of mosquito species. In the condition with wind blowing, do not conduct the test. Record wind velocity, temperature, relative humidity or rainfall for analysis, etc.

Table 2. Example of Randomization of 4 Doses of Repellent Sample, Positive and Negative Controls in 6 Subjects in 6 Collection Places

Test Onset Time at Collection Place(Hour)	Observation Time (Hour)[2]	Collection Place					
		1	2	3	4	5	6
0	0 - 1	*V5CN*	*V4D4*	*V6D2*	*V1D3*	*V2CP*	*V3D1*
1	1 - 2	*V4D4*	*V6D3*	*V5D1*	*V2D2*	*V1CN*	*V3CP*
2	2 - 3	*V2D1*	*V4CN*	*V1D4*	*V3D2*	*V6CP*	*V5D3*
3	3 - 4	*V1D2*	*V5CN*	*V2D3*	*V6D1*	*V4D4*	*V3CP*
4	4 - 5	*V2D3*	*V5CN*	*V1D1*	*V6D2*	*V3D4*	*V4CP*
5	5 - 6	*V4CP*	*V3D2*	*V5D4*	*V2D3*	*V1CN*	*V6D1*
6	6 - 7	*V4D1*	*V3D3*	*V5D2*	*V2CN*	*V6CP*	*V1D4*
7	7 - 8	*V1CN*	*V2D1*	*V4D2*	*V6D3*	*V5D4*	*V3CP*
8	8 - 9	*V5D4*	*V4D1*	*V3D2*	*V1D3*	*V6CN*	*V2CP*
9	9 - 10	*V3D2*	*V6CN*	*V5CP*	*V1D3*	*V2D4*	*V4D1*

[2] Observed at 15, 30 and 45 minutes during each observation period in each collection place.

D repellent concentration, CN negative control, CP positive control, V subject

<Calculation>

As test results, each dose in every repeated test, and the mean of the number of mosquitos collected within each observation period (that is, 15 ~ 16 min, 30 ~ 31 min, and 45 ~ 46 min) should be calculated.

The repelling effect (p) for each test period and dose should be calculated as follows.

$$p = 1 - (T / C) = (C / T) / C \qquad ②$$

Here, T is the number of mosquitos collected from the subjects treated with the repellent sample, and C is the number of mosquitos collected from the subjects in negative (or positive) control.

The persistency of the repelling effect can be measured from effective median dose (ED_{50}) and half-life of the repellent. The end point of the repellent can be evaluated by applying data to probit-plan regression model, which can be related to defensive effect of the repellent and natural logarithm (ln) of the applied repellent dose during the test period.

$$\ln [p / (1 - p)] = a + b_1(D_0) + b_2t_1 \quad ③$$

Here, p is the repelling effect, D_0 is the value of applied dose calculated by natural logarithm (ln[dose]), and t is the time after treatment. a, b_1 and b_2 are the coefficients calculated by using probit-plan regression model.

ED_{50} is the applied dose with 50% repelling effect (p = 0.5) at the repellent application time (t = 0). In the above formula ③, p is 0.5, t has 0, and when the formula is solved for D_0, the estimate of ED_{50} comes out. (See example A).

Other percentage should be set t = 0, and p can be calculated for specific applied dose. For example, to calculate ED_{90} using the coefficients presented in example A, add t = 0, p = 0.90 in formula ③ to calculate (see example B).

Other percentage such as median of efficacy time (ET_{50}) for a specific applied dose (D_0)(see example C) can be calculated in formula ③.

The half-life of the repellent can be calculated by using formula ④ as below.

$$\ln [1 / 2] b_1 / b_2 \quad ④$$

The CPT for the given dose can be calculated with the elapsed time to the initial mosquito landing or bloodsucking through each repeated test. The median and confidence interval of the CPT can be calculated using Kaplan-meier survival function procedures (see Attachment 2).

Example A

Evaluate the repelling effect of deet on *Anopheles gambiae s.l* by probit-plane regression model with the following coefficients.

a = 8.160

b_1 = 2.209

b_2 = -0.532.

ED_{50} can be calculated as follows.

(1) ln $[p/(1 - p)] = a + b_1(D_0) + b_2t_1$

(2) ln $[0.5/(1 - 0.5)] = 8.160 + 2.209(D_0) + -0.532\ (0)$

(3) ln $(1) = 8.160 + 2.209(D_0) + 0$

(4) ln $(1) = 0 = 8.160 + 2.209(D_0)$

(5) $-8.160/2.209 = D_0$

(6) $-3.694 = D_0$

(7) ln $(ED_{50}) = D_0$

(8) $ED_{50} = \exp(D_0)$

(9) $ED_{50} = 2.7183^{(-3.694)}$

(10) $ED_{50} = 0.025$ mg/cm^2

Example B

ED_{90} can be calculated as follows.

(1) ln $[p/(1 - p)] = a + b_1(D_0) + b_2t_1$

(2) ln $[0.9/(1-0.9)] = 8.160 + 2.209\ (D_0) + -0.532\ (0)$

(3) ln $[9.0] = 8.160 + 2.209\ (D_0)$

(4) $2.197 - 8.160 = 2.209\ (D_0)$

(5) $-5.963 = 2.209\ (D_0)$

(6) $-5.963/2.209 = D_0$

(7) $-2.699 = D_0$

(8) ln $(ED_{90}) = D_0$

(9) $ED_{90} = \exp(D_0)$

(10) $ED_{90} = 2.7183^{(-2.699)}$

(11) $ED_{90} = 0.067$ mg/cm^2

Example C

When the subject applied 0.50 mg/cm^2 (i.e. D_0 = ln [0.50] mg/cm^2) in his or her arm, the determination of ET_{99} (Use the same coefficients provided in example A)

(1) ln $[p/(1 - p)] = a + b_1(D_0) + b_2t$

(2) ln [0.99/(1.00 - 0.99] = $a + b1(D_0) + b_2t$

(3) ln (99) - 8.160 - (2.209 × -0.693) = $(-0.532)t$

(4) 4.595 - 8.160 - (1 1.531) = $-0.532t$

(5) -2.034 = $-0.532t$

(6) t = (-2.034/-0.532) = 3.8 hours (after application)

3.2 Efficacy and Persistency of Agent

<Overview>

This test is to measure the repelling power and CPT of the final agent produced by selecting the optimal effective dose of the repellent sample as the result of laboratory and outdoor test measurement through outdoor test.

<Test Preparation>

To minimize the difference of the number of mosquitos landing or bloodsucking by place before this test, a preliminary survey to evaluate the adequacy of the test place by collecting mosquitos landing in humans is necessary. With the preliminary survey, a place with uniform mosquito population with adequate bloodsucking rates can be selected. An adequate number of subjects who are trained for mosquito collection including subjects participating as positive control and persons who participate as negative control (for purpose of observing the degree of bloodsucking of mosquitos) without application of repellent should participate in this test. In this test, a minimum number of subjects with statistical significance should participate because of ethical reason.

<Test Method>

In a single test (1 day) in 1 place, the operator applies 1 mL each of the agent [or as positive control, 20% N,N-diethyl-3-methylbenzamide(Deet) ethanol solution] per about 600 cm^2 of skin in the exposed arm or leg of each subject. In terms of adhesive mosquito repellent, attach the agent in the test areas (wrist or ankle) according to the dose and administration of the applicable product. After drying the arms of each subject, record the application time. Locate the subjects in the test place randomly so that they can have distance of 20 m and more each other. The exposure time to mosquito for the subjects treated with the repellent and positive control in the collection place should be selected adequately for the target mosquito species. For example, if the subjects are bitten seriously, observe them for 1 minute each in 15 minutes from the application of the repellent (that is, 15 ~ 16 minutes), through 15, 30, and 45 minutes in 1 hour of observation period (0 ~ 1 hour) for 1 hour of the first measurement time. Regarding the adequate number of additional subjects (negative control), the degree of bloodsucking should be observed during the test. All mosquitos landed in the exposed arms or legs of the subjects should be collected with an aspirator, the number should be counted, and kept in a container to distinguish the species. The single (1 day) test should be conducted by changing the location of collection for each of subject, positive control and negative control randomly.

The collection of mosquito collected in humans should be conducted during the time in which the target mosquito species acts for bloodsucking, and the repellent should be well treated so that the test can be completed within the bloodsucking activity period of the target species. The test should be conducted until the time mosquitos landed at first or suck blood in the subjects.

<Calculation>

The complete protection time (CPT) is calculated with the repellent application time and the elapsed time (min) mosquito is landed for the first time or sucks blood. The result may be analyzed using Kaplan-Meier survival function (see 2.2. Calculation) and CPT median, and the applicable standard errors.

The percentage (%p) of repellent efficacy in outdoor test can be determined by each test time as follows.

$$\%p = ((C - T) / C) \times 100 \qquad ⑤$$

Here, T refers to the average number of mosquitos collected from the subjects treated with repellent in the test period, and C refers to the average number of mosquitos collected from negative or positive control subjects in the same period. To record the change of repellent percentage by time during 12 hours of test period, repeat calculation by each time.

Attachment 1.

○ The surface area of the skin in arms for repellent efficacy test can be measured by approximate value based on the cylindrical surface area. To calculate the surface area, the length of treatment area, distal treatment area, and proximal length of boundary are required.

○ The repellent should be applied evenly to overall area of the arm from wrist to elbow(A). With regard to the treated surface area, calculate the approximate value by measuring the wrist(B) circumference (cm), the circumference of elbow when the arm is stretched (cm)(c) and the distance (cm) from the elbow to wrist (D).

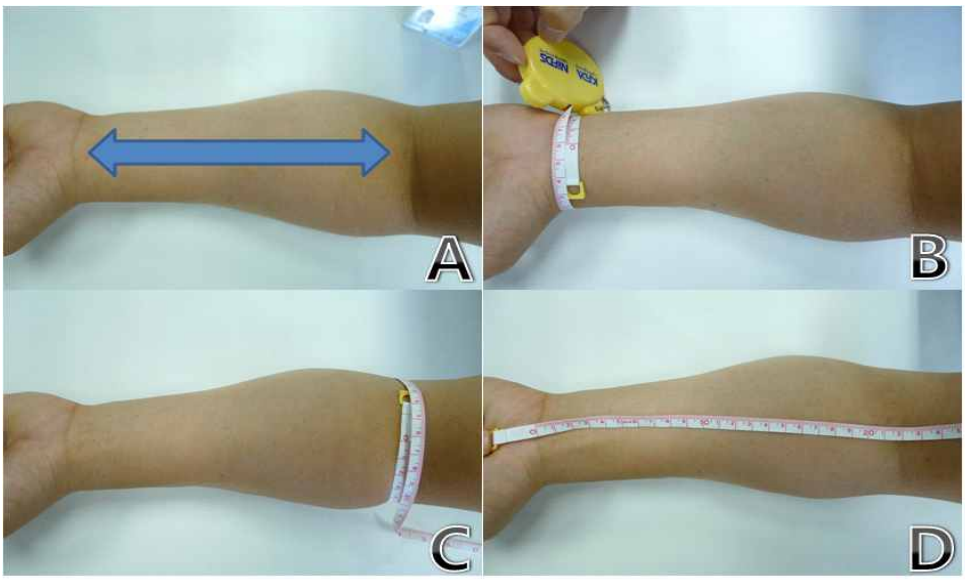

Figure 1. Repellent treatment area (A), wrist circumference measurement (B), elbow circumference (C), and length of arm (D)

○ The skin surface area (cm^2) should be calculated as follows.

$$\text{Surface area} = 1/2\ (C_w + C_e)\ D_{we}$$

C_w is wrist circumference (cm), C_e is elbow circumference (cm), D_{we} is the distance between C_e and C_w

<Example> Repellent test using the arm

When the subject's wrist circumference is 18 cm, elbow circumference is 27 cm, and the distance between the wrist and elbow is 26 cm, the operator can calculate as follows using the above formula.

Surface area = 1/2 (18 cm + 27 cm) x 26 cm

= 1/2 (45 cm) x 26 cm

= 22.5 cm x 26 cm = 585 cm^2

Attachment 2.

○ Kaplan–Meier function is a non-parametric statistics used to calculate CPT and the applicable confidence interval. CPT "survival function" should be determined based on CPT recorded during the test. The data required at this time are as follows.

CPT (min)(t) of each test (i) for repeated number (b)
(that is, t_i [i = *1, 2,... n*]).
Repeated number (r) of the test with the same CPT (j)
(here, $r \leq n$ (i.e., t_j [j = 1, 2, ... r])).
Repeated number of test not sharing the same CPT (that is, [$n - r$]).

To prepare the survival function measurements table, t_j time should be aligned in ascending order. For each t_j, n_j observed measure with bigger CPT than t_j may exist. The time interval between tj와 t_j+1 should be marked d_j, CPTs. Kaplan–Meier statistics can be estimated as survival probability, (or time for protection failure in opposition) $(n_j - d_j)/ n_j$. The standard error of the survival function estimates in time t can be defined as $s.e.\{S(t)\}$.

t(50), median of CPT is recorded at the top 50% of CPT (e.g., $S\{t(50)\} = 0.5$). The estimated CPT median is the smallest CPT of which $S(t) \leq 0.5$. 90% confidence interval of this estimate is suggested as $t(50) \pm 1.96\ s.e.\{t(50)\}$.

<Example>

If the recorded CPT of 48 subjects in virtual test of the efficacy of the repellent applied in human skin for 10 hours is described in Table 1, CPT for landing or bloodsucking in the remaining 11 subjects before the end of the test period (10 hours).

Table 1. Summary of Frequency of CPT of Repellent Treated in Human Skin

CPT (min)	120	150	210	390	420	450	480	510	600
Frequency (*dj*)	1	3	3	10	7	4	4	2	3

Based on this test result, estimates of $(n_j - d_j)/n_j$ and $S(t)$ and their standard errors as in Table 2.

Table 2. Calculation of $(n_j - d_j)/n_j$, $S(t)$ and Their Standard Errors

Time Interval (min) (tj)	nj	dj	$(nj - dj)/nj$	$S(t)$	Standard Error S(t)
0 ~	48	0	1.0000	1.0000	0.0000
120 ~	47	1	0.9787	0.9787	0.0210
150 ~	44	3	0.9318	0.9120	0.0420
210 ~	41	3	0.9268	0.8453	0.0538
390 ~	31	10	0.6774	0.5726	0.0798
420 ~	24	7	0.7083	0.4056	0.0776
450 ~	20	4	0.8000	0.3245	0.0719
480 ~	16	4	0.7500	0.2434	0.0643
510 ~	14	2	0.8571	0.2086	0.0597
600	11	3	0.7273	0.1517	0.0516

The median of CPT is the smallest CPT of which $S(t) \leq 0.5$. In t his case, it is t_j = 390, $S(t)$ = 0.5726, and t_j = 420 $S(t)$ = 0.4056. In this example, the maximum estimate of the CPT median is $t(50)$ = 420 min.

To calculate 95% confidence interval, set $S\{upper(50)\}$ =390, $S\{lower(50)\}$ = 420. The function of probability density in $t(50)$ can be calculated as follows.

$$f\{t(50)\}=[S(390)-S(420)]/[420-390]=[0.5726-0.4056]/30=0.005567$$

The standard error of survival function and standard error of CPT median in $t(50)$ should be calculated as follows

$$s.e.\{t(50)\}=(1/0.005567)\ X\ 0.0776\ =\ 13.94$$

In this example, 95% confidence interval of the estimated CPT median for this repellent is 13.94, ranged in 420 ± 1.96. Based on this, CPT median in 95 out of 100 observed values is expected to between 393 min and 447 min.

4. Reference

1) A Study on Efficacy of Mosquito Repellent, KFDA, Kim, Young-bong (2011)

2) Guidelines for efficacy testing of mosquito repellents for human, WHO/HTM/NTD/WHOPES(2009)