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Test Report

Laboratory study on the bite-preventing efficacy of One repellent formulation by Squito SARL in Arm-to-cage tests with yellow fever mosquitoes *(Aedes aegypti)* and three volunteers

- CONFIDENTIAL -

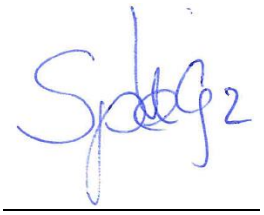
1. Executive Summary

The repelling and bite-preventing efficacy of one repellent formulation (TIZZY) was evaluated in Arm-to-cage tests with yellow-fever mosquitoes (*Aedes aegypti*) and three volunteers (female, 23 and 31 years and male, 25 years).

The sample was applied to a defined area on the forearm of the volunteer by weighting in the amount using precision scales. The treated arm was regularly exposed to caged populations of host-seeking test mosquitoes. The protection time until first confirmed bite (FCB= one bite followed by another one within the same test or in the consecutive test after 30 minutes) was documented. According to the European Chemicals Agency´s guidance documents on testing mosquito repellents, the FCB marks the end of complete protection time (CPT) and should be used as criterion for break-off (ECHA, 2021). The amount of product was approximated at 450mg per 100cm^2 of skin by the principal investigator using a video provided by the costumer.

The mean protection times were calculated at 5,5h

2. General Information

Sponsor	Squito SARL Chemin des Vernes 7 1936 Verbier, Switzerland
Testing Facility	Biogents AG Weissenburgstr. 22 D-93055 Regensburg
Principal Investigator	 signed Sergej Sperling, M Sc. Biology Date: 30.11.2023
Time Frame	Receipt of test formulations: 05.09.2023 Experimental start date: 10.11.2023 Experimental termination date: 17.11.2023 Data analysis and report: 30.11.2023

3. Material and Methods

3.1. Test Formulations & Application

The test formulation was provided by Squito SARL, Switzerland

The test formulation contained the following information:

[1] Tizzy; Gel anti mosquito (active ingredient: DEET 7%)

The test formulation was stored in its original packing at 20 – 22°C until the start of the test.

The amount of product was approximated at 450mg per 100cm^2 of skin by the principal investigator using a video provided by the costumer.
The amount was weighted in using precision scales and applied to a defined area on the forearm of the volunteer. Prior to the application, the skin was washed with fragrance-free soap, rinsed with water and wiped with 50% isopropyl alcohol. An area larger than the test window of the Arm-to-cage (ATC) (see 3.4) was marked to ensure that the exposed skin was entirely treated with repellent substance. The marked area had a size of ca. 100cm^2. Shortly after the application, the first efficacy test was conducted.

3.2. Test Mosquitoes *Aedes aegypti*

Female mosquitoes of the genus *Aedes* were reared according to the standard protocol at a temperature of 27 ± 0.5°C, a relative humidity of 65 – 80 % and a 12:12 hour photo period. The light period (450 Lux) was set from 8:00 to 20:00. After hatching from the eggs, larvae were kept in water basins (30 x 30 x 10 cm) filled with a 1:1 mixture of deoxygenized tap- and deionised water and fed with fish food flakes (Tetra Min®). Pupae were transferred to a cage (40 x 30 x 20 cm) for emergence, adult mosquitoes were provided with sugar solution (10% dextrose). Mosquitoes at an age of 7-12 days after emergence, that have never received a blood meal, were used for ATC tests.

3.3. Test Room

Cage tests were performed in climatized room of 41m^3 without windows. The temperature and relative humidity of the room air were set to 27 ± 1°C and 75 ± 5% RH. The room was illuminated with full spectrum LED light tubes (intensity 450 Lux).

3.4. ATC test cages

ATCs are an in-house improvement of conventional test cages for the evaluation of mosquito repellents (Obermayr et al., 2010). The cages have a volume of 27.000 cm³ (41 x 41 x 16 cm). Four sides of a cage are made of acrylic glass, the floor is made of metal sheet and the rear side is covered by a gauze sleeve. The floor sheet is equipped with a test window (size: 56 cm²; 14.8 x 3.8 cm) for the exposure of the treated arm. In between tests, ATCs are connected to a ventilation system that provides it with clean, warm and humid air (26 ± 1°C, 75 ± 10% r.H.) to remove remaining host odours and repellent volatiles from the air inside the cage.

Each cage is filled with populations of 30 mosquitoes, those are lured out of their rearing cages by a natural stimulus (human hand) to ensure that only host-seeking females are used for the repellent tests.

Fig. 2: ATC (side view). The air ventilation system is connected to the cage (black arrow). The arm is exposed at the test window in the metal floor sheet (black rectangle). The rear side is covered by gauze.

3.5. Test Procedure

3.5.1. Zero Control (Biting Activity)

Prior to an individual repellent efficacy test, the biting activity of the test mosquitoes was verified with the other, untreated forearm of the volunteer.
In order to keep the biting pressure on the untreated skin low, a modified spacer covered with fine mosquito netting was used during control tests (see fig. 2). In this way, mosquitoes are still attracted to the skin odours and land on the net, however, they are unable to reach the skin and pierce it.

Positive *Ae. aegypti* biting activity requires a minimum of 10 landings in 30 seconds. In case biting activity is lower, 5 to 10 new mosquitoes will be added to the cage or 30 fresh mosquitoes are used. The exact time until 10 landings are received was documented, this time value was used for the calculation of the protection percentage on the treated arm (see 3.5.2.).

The modified spacer is never used during tests of repellent treated skin, here mosquitoes are allowed to be in direct contact.

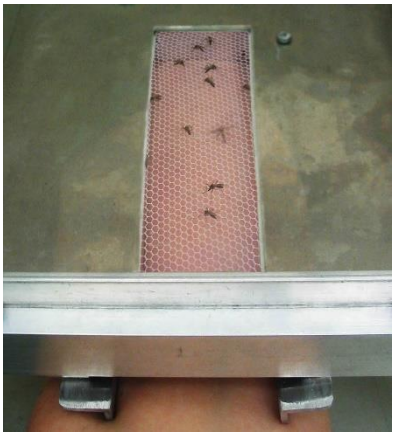


Fig. 3: Zero Control. Fine mosquito netting keeps mosquitoes from piercing the untreated skin.

3.5.2. Test Proper

ATC tests were performed following recommendations by two guidelines for repellent testing published by the *American Environmental Protection Agency* (EPA, 2010) and the *World Health Organization* (WHO, 2009).

The test formulation was applied to a defined area on one forearm of the volunteer. Repellent efficacy was verified for the first time shortly after product application and then again in regular 30 minutes intervals up to a maximum of 8 hours or until repellency failed. Each single test lasted 2 minutes, during this time the number of landings and bites on the treated skin were recorded.
Repellent efficacy was evaluated using the time until first confirmed bite (FCB= one bite followed by another one within the same test or within the consecutive test after 30 minutes). The FCB is defined to mark the end of complete protection time and is used as the criterion for break-off for repellent tests (according to the technical notes for guidance by ECHA, 2023).

Tests were conducted with three volunteers (female, 23 and 31 years and male, 25 years) against one mosquito species, the yellow fever mosquito *Ae. aegypti*. All volunteers was attractive to the test mosquito species, thereby meeting the requirements to participate in repellent efficacy studies.

Cages were connected to the air ventilation system in between single tests (zero control and repellent efficacy test) to avoid an accumulation of host odors and active ingredients inside the cage. Test mosquitoes that started to engorge blood during a test were replaced by new individuals to ensure that the number of host-seeking females stayed constant throughout the test day.

4. Results

4.1. Protection times against *Ae. aegpyti*

Test mosquitoes showed a reliable biting activity throughout the entire study, twenty probings on untreated skin were recorded after an average of 36,7 seconds (n = 33).

The protection times until first bite and FCB are shown in table 1.

Tab. 1: Mean protection times in ATC tests with *Ae. aegypti*. The protection time is shown for the test formulation. The protection time were generated from data sets provided by 3 volunteer (n = 3).

Test formulation	Mean protection [h] until **first bite**	Mean protection [h] until **FCB**
Tizzy	5h	5,5h

The test formulation "Tizzy" provided an average protection until first bite of 5h and until FCB of 5,5h after an evaluation with three volunteers.

5. Discussion

Laboratory cage tests are usually the method of choice to evaluate the contact- or bite-preventing potential of mosquito repellents. In contrast to field tests, which provide the most valuable information but are greatly influenced by a variety of abiotic and biotic factors (e.g. climatic conditions, mosquito population & density, activity patterns), laboratory cage tests can be performed at any time under standardized conditions and allow the use of laboratory-reared, pathogen-free vectors of diseases, which are important targets of personal protection measures.

Tests were performed with one mosquito species of medical importance. The diurnal yellow fever mosquito *Ae. aegypti* is very aggressive, has a broad activity pattern and can easily be maintained under laboratory conditions. Not only for these reasons this species is used as a standard mosquito for behavioral tests by research groups worldwide; *Ae. aegypti* is also the main vector of important arboviral diseases such as dengue and zika.

ATC tests were developed on the basis of two guidelines for repellent testing published by the EPA (2010) and the WHO (2009). The applied procedure is an improved version and introduces a few modifications to the conventional set-up in order to create more defined testing conditions and increase the reproducibility of the test (Obermayr et al., 2010):

ATCs are connected to an air ventilation system in-between single tests, by doing so the incoming warm and humid air prevents the accumulation of host odours and repellent substances inside the test cages. The use of a defined test area on the forearm, instead of using the entire forearm, also minimizes the entry of active substances into the test cage. Compared to conventional cages, test mosquitoes are exposed to fewer amounts of repellent for a shorter period of time which prevents from exhaustion and a decrease in biting activity. EPA and WHO protocols use hundreds of mosquitoes per test cage to compensate for mosquito exhaustion throughout the test day, with the air ventilation- and test window system ATCs require only 30 females, which also reduces density-related stress. The required control biting rate of 10 bites (landings) in 30 seconds is also achieved with 30 test mosquitoes.

Mosquito-density in the test cage and short distances to the treatment area may still lead to a higher biting pressure compared to natural conditions in the field. Thus, shorter repellent protection times can occur during laboratory cage test studies. However, a comparison ATC tests, conventional arm-in-cage tests and field tests revealed, that protection times obtained during ATC tests can be better related to field test data than protection times documented with conventional tests (Obermayr et al., 2010). ECHA (European chemical agency) included the test protocol in its guidance for repellent testing (**ECHA** (2023) Guidance on the Biocidal Products Regulation. Volume II Efficacy – Assessment and Evaluation (Parts B&C). Version 5.0, 2023)

Cage tests were performed with three volunteers. During control tests of untreated skin, it took an average of 15 and 49 seconds until 20 probings by *Ae. aegypti* were counted. The biting activity tests / attractiveness of volunteers do not allow to draw conclusions on the protective effects provided by a repellent. Thus, differences in individual protection times are more likely related to the skin properties of the volunteers (absorption & evaporation rates) and not so much to the individual attractiveness for the test mosquito species.

References

ECHA (European Chemicals Agency) (2023) Guidance on the Biocidal Products Regulation. Volume II Efficacy – Assessment and Evaluation (Parts B&C). Version 5.0, 2023

EPA (United States Environmental Protection Agency) (2008). Product Performance Test Guidelines. Insect Repellents to be Applied to Human Skin. 23. September 2008

Obermayr, U., Rose, A. and Geier, M. (2010). A Novel Test Cage with an Air Ventilation System as an Alternative to Conventional Cages for the Efficacy Testing of Mosquito Repellents. Journal of Medical Entomology 47 (6): 1116-1122 / DOI: 10.1603/ME10093

WHO (World Health Organization) (2009). Guidelines for Efficacy Testing of Mosquito Repellents for Human Skin. WHO/HTM/NTD/WHOPES/2009.4