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AN IN VITRO ASSAY FOR TESTING MOSQUITO REPELLENTS EMPLOYING A WARM BODY AND CARBON DIOXIDE AS A BEHAVIORAL ACTIVATOR

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ABSTRACT. We describe here an in vitro behavioral assay for testing mosquito repellents applied in a dose-based manner to a warm body (34°C) in test cages. The system was used to assess the sensitivity of 4–6-day-old *Anopheles gambiae* to the insect repellent diethyl methyl benzamide (deet). These tests were made in the absence and presence of additional carbon dioxide (CO_2) applied as a pulse to activate mosquitoes in the cages. In the absence of the CO_2 pulse the mosquitoes hardly responded to the warm body. Increasing the CO_2 level in the cage by 1,000 parts per million caused a 25-fold increase in the number of landings by mosquitoes on the warm body in 2-min tests. This mosquito activation allowed the measurement of a significant reduction in the number of landings to bite on the warm body with increasing doses of deet (0.4 to 3.8 μg/cm^2). An asymptotic nonlinear model fitted to the repellency data in the presence of CO_2 allowed estimation of the effective dose of deet that reduced landings to bite by 50% (ED_{50}) at 0.95 μg/cm^2 (5 nmol/cm^2) and the corresponding ED_{95} at 4.12 μg/cm^2 (21.5 nmol/cm^2). This in vitro bioassay has the advantage of permitting a fast throughput of test products under standardized conditions and is suitable for screenings designed for the purpose of discovering lead products with as yet unknown human toxicological and dermatological profiles.

KEY WORDS In vitro repellent assay, mosquito, insect vector, *Anopheles gambiae*, deet

INTRODUCTION

Diethyl methyl benzamide (deet) is the most widely used repellent against a wide range of biting arthropods including mosquitoes (Debboun et al. 2007), although new N-acylpiperidine products with longer lasting repellence against *Aedes aegypti* (L.) have been recently described (Katritzky et al. 2008). Estimation of repellency is usually based on comparison between the number of bites on a treated versus an untreated forearm or leg exposed to mosquitoes for a finite period (Granett 1940). The repellent efficacy of a product is estimated as the rate of bites per minute or the time required to obtain the first bite (protection time) on an individual from a test population of mosquitoes. To evaluate the effective dose (ED) required to repel a given percentage of an ectoparasite test population, a ''dose-response method'' has been adopted where several doses of a repellent can be tested simultaneously on a test subject's limb exposed to mosquitoes (Buescher et al. 1982, Klun and Debboun 2000). However, in vivo experiments are not always ideal, since mosquito attraction can vary between test subjects (Carlson et al. 1992). In addition, pharmacokinetic studies on the skin of different mammals revealed that portions of the initial dose of deet applied, which ranged from 4 to 500 μg/cm^2, were absorbed over a time interval of 10 h to 5 days (Qiu et al. 1998) and, as such, did not evaporate from the skin. Although repellents designed to protect humans must eventually be evaluated in vivo, this is not necessarily the case for screenings designed for the purpose of discovering products with as yet unknown human toxicological and dermatological profiles. Bar-Zeev and Schmidt (1959) were the earliest to use a membrane, derived from the outermost layer of ox cecum, to evaluate repellent product efficiency. Subsequently, the Klun and Debboun module (2000) was adapted for in vitro tests on repellents using Baudruche® and collagen membranes (Klun and Debboun 2005).

In this study, we recorded in the test cages the number of landings by female *Anopheles gambiae* Giles, activated by a CO_2 pulse, on a warm body treated with increasing doses of deet and showed that the number of mosquitoes landing decreased with an increasing dose of the repellent. This in vitro assay, which uses a dose-based approach, can be readily adopted in screenings to identify novel repellent molecules and to establish threshold levels above which such molecules can affect mosquito behavior.

MATERIALS AND METHODS

Mosquitoes: The *An. gambiae* colony (16cSS strain, derived in 1974 from wild caught adults originating from Lagos, Nigeria, West Africa) was maintained in a climate chamber (28°C, 80% relative humidity [RH]) under a 12:12 light:dark cycle with 2 h simulated sunrise and sunset. Females were fed on a Guinea pig (*Cavia*

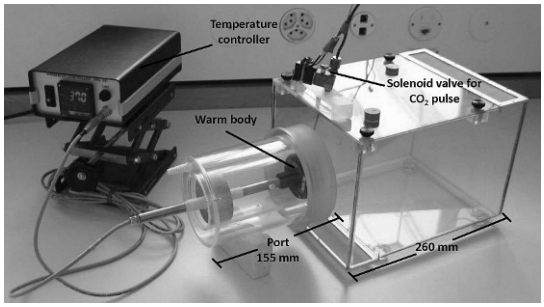

Fig. 1. The bioassay setup with the mosquito cage, warm body inserted into the port, temperature controller and solenoid valve for CO_2 pulse delivery.

porcellus L.) once a week and eggs were recovered on wet filter paper. About 250 larvae were reared in trays with 400 ml distilled water (8 mm deep) and fed with pulverized Tetramin® fish food. About 800 adult mosquitoes emerged into rearing cages (350 × 350 × 550 mm high) and were provided with 10% sucrose and water ad libitum. For the bioassay, we used 50 ± 6 *An. gambiae* females between 4 and 6 days old that were attracted to a hand in the rearing cages. Jones and Gubbins (1978) have shown an increasing flight activity of inseminated *An. gambiae* females during the scotophase from 4 days old. Bioassay cages containing the mosquitoes were positioned at least half an hour before the tests in the experimental climate chamber, to avoid mechanical stimulation of the mosquitoes through handling of the cages before tests.

Bioassay cages: The bioassay cages were made of polycarbonate (Makrolon®; Borotec, Bern, Switzerland; 200 × 260 × 180 mm high; Fig. 1). They had a front side opening (110 mm diameter) in which a port was fitted permitting the introduction of the warm body (below) in line with the wall of the cage without escape of mosquitoes. The port was made of 2 concentric acrylic glass Gevacril® tubes (110 mm outside diameter, 63 mm inside diameter, and 155 mm long; Melzo, Italy) into which the warm body was inserted during 2-min experiments. Otherwise a plastic "stopper" was put into the port. Two openings, 1 at the bottom and 1 on the top of each cage (200 × 50 mm), closed by netting (1 mm mesh size) facilitated exchange of air.

Warm body: Attraction of female mosquitoes was evaluated using a cylindrical warm body (WB, 60 mm diameter disk, 20 mm thick) as a heat source (Fig. 1). A low voltage electrical current (power 33 Watts) was split in parallel over 3 resistors fixed in a triangular arrangement to the inner wall of the black anodized aluminum corpus of the WB. A glass Petri dish (60 mm diameter) with a sandblasted floor was attached to the face of the warm body by metal springs. A PT100 temperature controller maintained the temperature of the WB to within 37°C ± 0.5°C, assuring a temperature of 34°C ± 0.5°C on the surface of the Petri dish. A white filter paper disk (55 mm diameter, Whatman No. 10 311 807) was inserted between the Petri dish base and the black warm body surface to visualize mosquito landings.

Bioassays: Experiments were conducted in a walk-in climate chamber (25°C ± 1°C and 80% ± 3% RH) during the last 6 h of scotophase. The sides of the bioassay cages were covered with white cardboard to avoid activation by visual stimuli due to the presence of the experimenter. Dim light (4 Lux) was provided from above by fluorescent tubes (Philips TLD, 32 Watts at 36 KHz). The Petri dish floor was treated with 100 µl pure ethanol (Merck, Darmstadt, Germany) as control or with 100-µl ethanol solutions containing of 0.1, 0.3, or 1 µg/µl of deet (Riedel de Haen, Pestanal®, Seelze, Germany) providing doses of 0.4, 1.1, and 3.8 µg/cm^2 on the Petri dish floor. After evaporation of the solvent (40 s) the warm body with the Petri dish attached vertically to its face was introduced through the port such that the Petri dish base was in line with the vertical wall of the cage.

In the case of experiments performed in the presence of CO_2, a 0.2-sec pulse of pure CO_2 (volume 6.25 ml, 99.99%, $H_2O \leq 200$ parts per million [ppm]) from a pressurized cylinder (Carbagas, Switzerland) was applied using a solenoid valve just after the warm body with the attached Petri dish was introduced (Fig. 1). This caused the CO_2 level in the cage to rise from 500 ppm to 1,500 ppm to activate the mosquitoes. Such an increment ensured that all the mosquitoes in the cage were activated. The CO_2 pulse was delivered 40 mm above the center of the warm body and at 10 mm in front of it. CO_2 levels were measured using a gas analyzer (model Li-820; LiCor, Lincoln, NE) near the wall on the opposite side of the cage and at 90 mm from the top. CO_2 values were measured during each experiment at 1-sec intervals and stored on a personal computer. To monitor the number of landings, the warm body was filmed with a low light sensitive black and white charge-coupled device camera (model WV BP310; Panasonic, Osaka, Japan) equipped with a TV zoom lens (model J6 × 12, 12.5–75 mm 1:18; Canon, Tokyo, Japan) placed at the opposite side of the cage.

Doses of deet at 0.4, 1.1, and 3.8 µg/cm^2 (2, 6, and 20 nmol/cm^2, respectively) were tested without an additional CO_2 pulse in 12, 14, and 18 cages, respectively. These 44 tests without the addition of CO_2 were preceded by ethanol controls. The same doses of deet were also tested in the presence of the CO_2 pulse in 12, 17, and 22 cages, respectively, preceded by a test with ethanol as control with a CO_2 pulse in all 51 cages. The minimum time interval between a

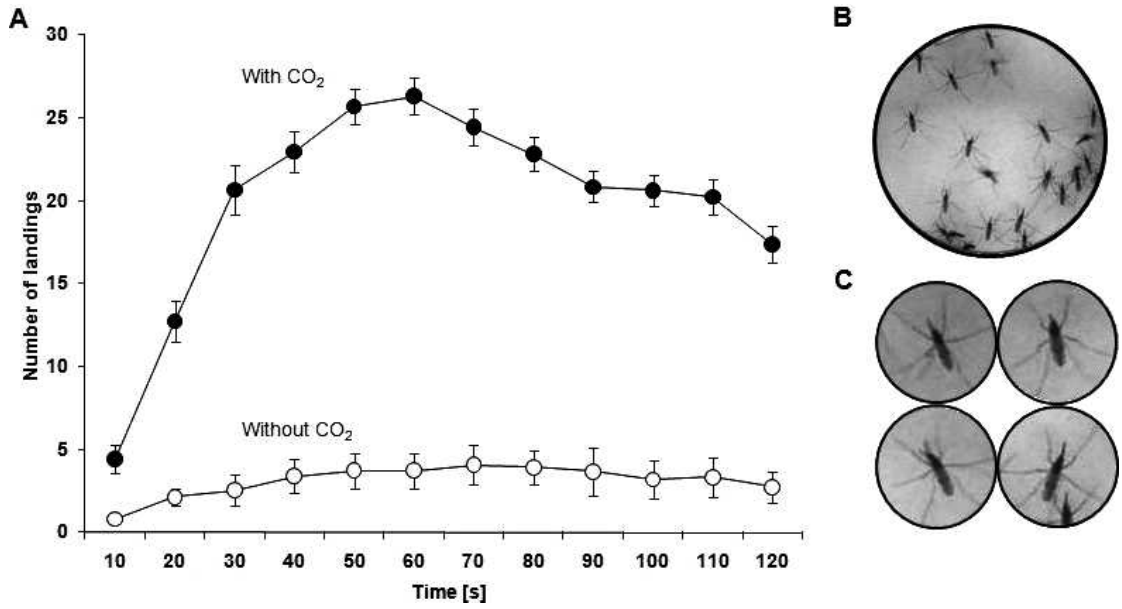

Fig. 2. (A) Mean number of landings made by *An. gambiae* females at 10-sec intervals on the warm body heated at 34°C in the presence of a CO_2 pulse in cages with 50 ± 6 mosquitoes (n = 39 cages, solid circles) compared with the number of landings on the warm body in absence of the CO_2 pulse (n = 15 cages, open circles). The presence of CO_2 as an activator causes significantly increased visits by *An. gambiae* to bite on the warm body over the first 30 sec; bars = 1 SE. **(B)** *An. gambiae* landing on the WB surface. **(C)** Different proboscis positions depicting probing attempts by *An. gambiae* females on the WB surface in the presence of CO_2.

control and a deet treatment was never shorter than 20 min for a batch of mosquitoes. Dekker et al. (2002) allowed *An. gambiae* to acclimatize for 20 min in an olfactometer before testing. The different doses of deet were tested at random, and each cage was used only once for each dose. All cages were furthermore exposed to the WB plus a CO_2 pulse after a series of the aforementioned experiments to confirm that the mosquitoes still showed appetence (data not shown).

Data analysis: The total number of mosquito landings over 2 min on the vertical surface of the Petri dish was counted for ethanol controls and for the 3 doses of deet tested with and without the CO_2 pulse. In addition, we counted the number of mosquito landings in controls on the Petri dish treated with ethanol alone on the warm body for 10-sec intervals during 2 consecutive minutes in 39 cages in the presence and in 15 cages in the absence of the CO_2 pulse. Most of the mosquitoes left the warm body after 1 to 15 sec but landed again after a short period of time elsewhere on the Petri dish floor. As such, multiple landings by the same mosquitoes were counted as independent events.

For each cage, a repellency index for deet was calculated using the formula

$$\frac{L_{\text{control}} - L_{\text{deet}}}{L_{\text{control}}} \times 100$$

where L was the number of landings on control or the test surfaces. An asymptotic nonlinear model

$$\text{repellency} = 100 \times \{1 - \exp[-\exp(A \times \text{dose})]\}$$

using an equation passing through the origin (no repellent effect when no deet was applied) and with a fixed asymptote converging to 100% repellency was fitted to the data set of repellency indices recorded for each cage with a nonlinear regression (NLS in R version 2.9.0). This software was also used for graphical representation of data.

RESULTS

In the presence of the WB alone the visit rate by *An. gambiae* females reached a mean level of 3 per 10-sec interval with 10% to 60% of the mosquitoes in each cage responding, and this barely changed over the 2 min of observation (Fig. 2A open circles). Injection of a pulse of CO_2 into the cage induced a drastic increase in the number of mosquitoes visiting the WB over 30 sec to reach a plateau at a mean level of 22 visits per 10-sec interval with 95% of the individuals responding to the warm body (Fig. 2A solid circles). Remarkably, most landings on the Petri dish base were immediately followed by several attempts of probing with mouthparts (Fig. 2B, 2C), similar to the biting pressure observed in tests on human skin. Probing attempts were too difficult to count accurately.

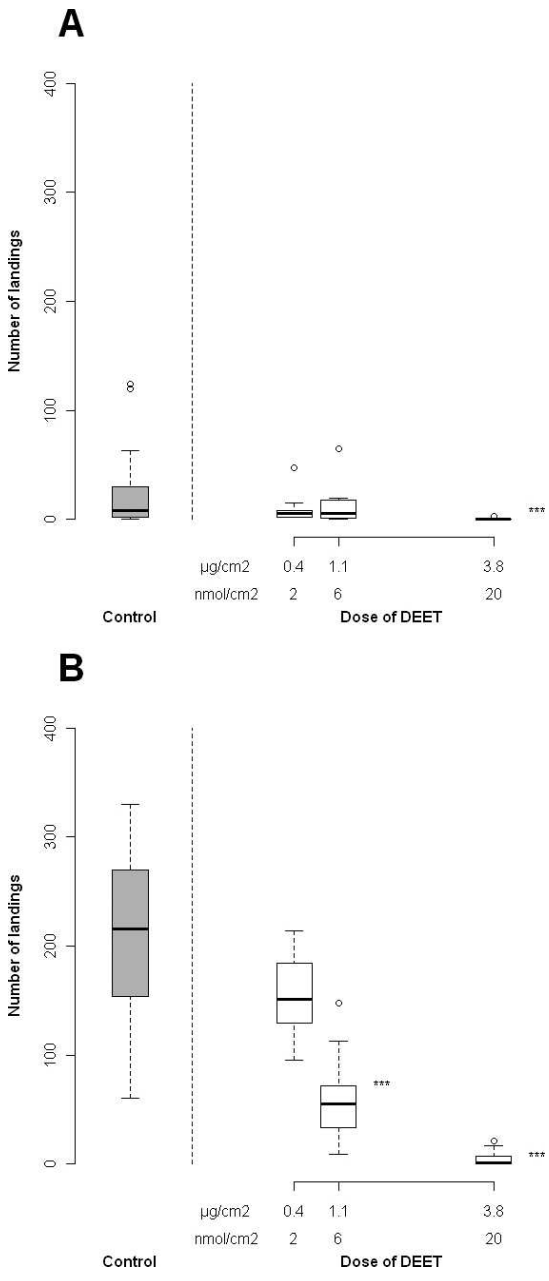

Fig. 3. Number of landings during 2-min experiments made by female *An. gambiae* on the warm body treated with increasing doses of deet (A) in absence and (B) in presence of a CO_2 pulse. Controls (0 µg/cm^2) are depicted in gray, and the 3 different doses of deet tested are shown in white. Box plots represent the median (black bar) and the 25%–75% interquartile interval (box), whiskers depict the 10th–90th percentile, and the points depict outliers. *** Doses different from the control at $P < 0.001$.

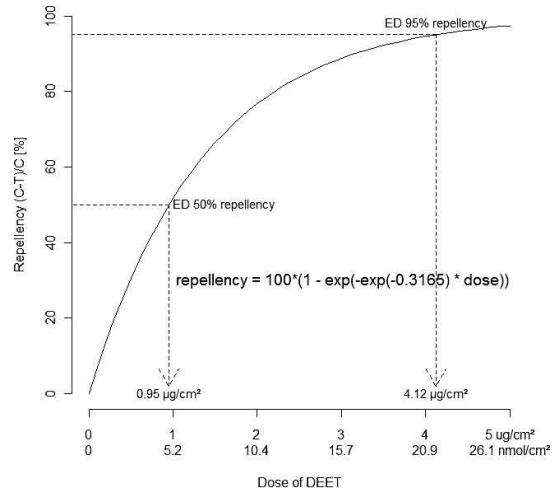

Fig. 4. Dose-response curve describing the repellency of deet on a warm body to *An. gambiae* females. The curve was fitted using an asymptotic nonlinear regression model passing through the origin. The arrows indicate the estimates of the effective doses that cause 50% and 95% repellency.

In treatments with deet but without the CO_2 pulse, the cumulative number of females landing on the WB treated with doses of 0.4 or 1.1 µg/cm^2 of deet were not different from the control (Kruskal–Wallis test, $P > 0.05$). Only at 3.8 µg/cm^2 was a significant drop in landings established (Mann–Whitney *U*-test, $P < 0.001$; Fig. 3A). In the presence of the CO_2 pulse, female *An. gambiae* showed a clear dose-dependent reduction in the number of landings in response to deet (Fig. 3B). A strong interaction between CO_2 level and deet concentration was confirmed by a Friedman 2-way analysis of variance ($P < 0.05$). The 0.4 µg/cm^2 dose of deet approached the lower amount of this product that affected landings by female *An. gambiae* on the WB (Mann–Whitney *U*-test, $P = 0.32$, compared to the control; Fig. 3B). The 1.1 µg/cm^2 dose induced a 4-fold reduction in the median number of landings in the presence of the CO_2 pulse (Mann–Whitney *U*-test, $P < 0.001$; Fig. 3B). The response of *An. gambiae* to the WB treated with the highest dose of deet in the presence of the CO_2 pulse was even higher compared with the situation without the CO_2 pulse (Mann–Whitney *U*-test $P < 0.001$; Fig. 3A and B). Despite this response, the median number of landings on the WB with CO_2 added was only 1 per 2-min experiment at the 3.8 µg/cm^2 dose (Fig. 3B).

The nonlinear asymptotic regression model indicates a highly significant dose-dependent effect of deet on *An. gambiae* in the presence of an augmented level of CO_2 (natural log rate constant 0.3165, *t*-value 2.957, residual standard error 19.82, df 50, $P \leq 0.01$; Fig. 4). The effective

dose to reduce landings by 50% (ED_{50}) was estimated at 0.95 μg/cm^2, and the corresponding ED_{95} at 4.12 μg/cm^2.

DISCUSSION

The mosquitoes increased their visits on the WB heated to 34°C when the CO_2 level increased by some 1,000 ppm in the bioassay cage. We chose to add 1,000 ppm CO_2 to assure adequate stimulation (Grant et al. 1995). This permitted the establishment of a clear dose-dependent response by *An. gambiae* females to the different doses of deet tested in the presence of the CO_2 pulse. In the absence of CO_2 as an activator, the WB was largely disregarded even in controls. Although heat is important for the approach of mosquitoes to a nearby host (Khan et al. 1966, Gillies and Wilkes 1969), this physical cue alone only elicited a response from a few individuals in our cages.

Khan and Maibach (1966) and Khan et al. (1966) have already shown that attraction to a WB heated to 34°C is increased for *Ae. aegypti* when CO_2 is released in its vicinity. A brief CO_2 pulse can increase sensitivity to skin odors in female *Ae. aegypti* and increase the number that find this odor source in a wind tunnel (Dekker et al. 2005). Furthermore, Mayer and James (1969) have demonstrated in an olfactometer that adding 500 ppm CO_2 increases the percentage of *Ae. aegypti* attracted to an arm treated with 1 ml of 5% deet by 3-fold. The 3.8 μg/cm^2 dose of deet showed a high repellency index against *An. gambiae* in our assay even in the presence of the CO_2 pulse. We also performed trials with *An. stephensi* Liston and *Ae. aegypti* using this assay and found these 2 species show responses similar to *An. gambiae*. At 3.8 μg/cm^2, the repellency index of deet reached 74% for *An. stephensi* and 94% for *Ae. aegypti*.

Our results confirm the effectiveness of deet in repelling mosquitoes. Its effect is related to the dose applied, with increasing doses causing decreasing numbers of *An. gambiae* landings on the WB in the presence of the CO_2 stimulus. The response curve established from our experiments suggests that the dose of 0.4 μg/cm^2 (2 nmol/cm^2) is near the lower amount of this product that affects the landing behavior of female *An. gambiae* on the WB. The effective dose that repels 50% of a test population of *An. gambiae* (ED_{50}) on rabbits has been estimated at 2.5 μg/cm^2, and the ED_{95} has been estimated at 12.8 μg/cm^2 (Robert et al. 1991). The ED_{95} for biting reduction by *Ae. aegypti* was estimated at 23 nmol/cm^2 (4.4 μg/cm^2) on human volunteers (Klun et al. 2004). The effective doses of deet required for repellency on humans are higher, probably because of the anthropophilic preferences of species such as *An. gambiae*. Indeed, the ED_{50} of deet for the same 16cSS strain of *An. gambiae* used here has been estimated at 19.9 nl/cm^2 (19.82 μg/cm^2) and the ED_{90} at 71.2 nl/cm^2 (70.92 μg/cm^2) for humans (Curtis et al. 1987). This means that the dose inhibiting 95% of the landings in our assay (4.12 μg/cm^2) is some 20 times lower than that required to affect 90% of individuals in an *An. gambiae* population attempting a blood feeding on humans. This difference is probably due to the fact that the WB does not carry the numerous chemicals associated with the skin of the vertebrate host. We have compensated for this lower attractiveness of the WB by releasing CO_2 to simulate the increasing concentration of this metabolite encountered by mosquitoes near a potential human host that releases about 45,000 ppm CO_2 at each expiration (Gillies 1980).

There is a range of in vivo tests involving volunteers to evaluate repellence as described by the US Environmental Protection Agency (US EPA 1999) and the one used recently in development of novel repellents for mosquitoes by Katritzky et al. (2008). The bioassay described here has the advantage of permitting a faster throughput of test products under standardized conditions. The rearing and test condition for this study were such that there was no shift in the phenological state of the mosquitoes over the period of the study, i.e., adult emergence and daily activity rhythm were constant. In addition, the experimental design involved controls on each day of tests.

This assay is already being used to test products affecting *An. gambiae* behavior in the framework of the EU-sponsored European Network for Advanced Research on Olfaction for Malaria Transmitting Insect Control (www.enaromatic.org).

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