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A WIND TUNNEL BIOASSAY SYSTEM FOR SCREENING MOSQUITO REPELLENTS

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ABSTRACT. A wind tunnel bioassay system to screen mosquito repellents is described. A wind tunnel is utilized to exploit the upwind flight response of host-seeking mosquitoes. Mosquitoes within the wind tunnel are activated with human breath, fly upwind, and land on heated chick skins. This behavioral sequence results in a consistently high percentage of the test population approaching repellent or control stimuli. The bioassay system is calibrated with diethyl methylbenzamide against *Aedes aegypti* and demonstrates a reproducible dose–response relationship. The persistence of diethyl methyl benzamide after a 1-h period is also recorded. The design of the bioassay system permits simultaneous, independent testing of 3 candidate repellents. The wind tunnel bioassay system is compared to other techniques for evaluating mosquito repellents.

KEY WORDS Mosquito, *Aedes aegypti,* repellent, diethyl methylbenzamide, wind tunnel

INTRODUCTION

Deet (diethyl methyl benzamide) was 1st reported as a mosquito repellent by McCabe et al. (1954) and is still regarded as a highly effective repellent. Deet does, however, have some unpleasant characteristics: it is a plasticizer, is often considered to have an unpleasant odor and feel, and has been associated with some toxic side effects (Gryboski et al. 1961, Miller 1982). Davis and Sokolove (1976) demonstrated that deet altered the response of lactic acid (an attractive component of human sweat) receptors on the antennae of *Aedes aegypti* (L.) mosquitoes. However, the structure–activity relationship of deet and other structurally unrelated but effective repellents has yet to be fully understood (Skinner and Johnson 1980). This comparative lack of knowledge has led investigators to search for new and more effective repellents to take the approach of screening large numbers of potential candidate compounds.

The human biting test is generally considered to be the most realistic method of evaluating the effectiveness of a compound. A human arm is treated with a known dose of the potential repellent and introduced to a cage of mosquitoes. Recordings are made of the number of mosquitoes attempting to bite and are compared to an untreated control arm. The effectiveness of the compound is assessed in terms of the observed reduction in biting between the treated arm and the control arm. The arm test is a comparatively slow method of testing. A limited number of compounds can be tested on an individual's arm, and time must be allowed for the treated arm to return to the control level of attractiveness. Using several individuals can introduce significant variation into the trial. The volunteers have to be prepared to be bitten by mosquitoes, especially during control studies. Finally, any compounds of unknown or suspect toxicity or irritancy have to be tested on animals prior to human testing.

To circumvent these problems, Bar-Zeev and Smith (1959) introduced the concept of evaluating repellents by treating artificial membranes that covered small reservoirs of heated blood. The effectiveness of the repellent was assessed in terms of the numbers of blood-fed mosquitoes in comparison to the amount of repellent applied. Rutledge et al. (1976) modified this technique by introducing mosquitoes into a cage that contained several wells of heated blood covered by membranes that were treated with a range of amounts of repellent. The system is considered to give the mosquitoes a free choice, as they are exposed to a range of amounts and can select membranes that have been treated with tolerable levels of repellent. Rutledge et al. (1976, 1985) consider that this design more closely represents the natural situation, in which a mosquito is free to select less well treated areas of skin or even an untreated host.

Buescher et al. (1982b) further modified this design by strapping a similar type of cage onto a human forearm. Buescher et al. (1982a) tested a range of repellents against *Ae. aegypti,* and this test procedure is recognized as a standard test method (Anonymous 1984). This technique has been used by Buescher et al. (1983) to examine the persistence of deet over a period of time, and Rutledge et al. (1985) have constructed mathematical models relating repellency to time and dose.

While conducting the human arm–biting test with deet and *Ae. aegypti,* we observed considerable variation between replicates. A major contributing factor appeared to be variation in the percentage of mosquitoes in the test population that actually responded to either the control or test arm. We decided to try to reduce this variation by designing a bioassay system that would result in a consistently high percentage of the test population responding to the control and repellent stimuli. A

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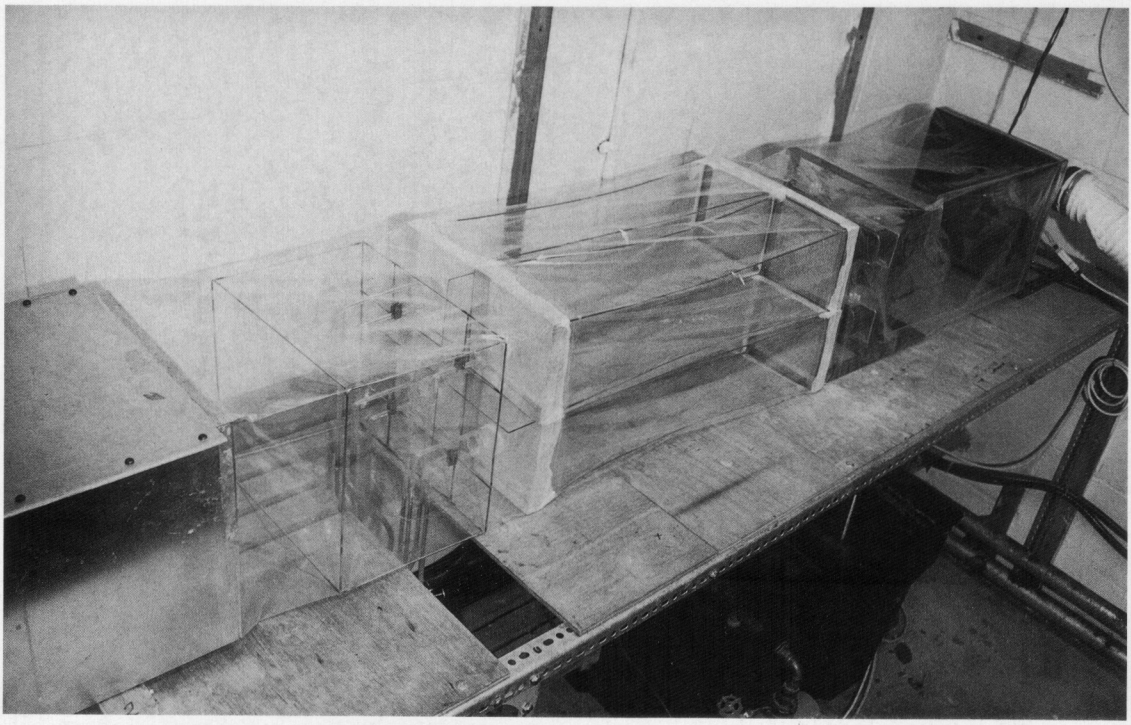

Fig. 1. Photograph of the bioassay system.

wind tunnel design was chosen so that an olfactory stimulus could be used to elicit the upwind flight response of host-seeking mosquitoes and bring them into contact with the test stimuli. In contrast to the free-choice bioassay design, the repellent doses were deliberately kept independent.

MATERIALS AND METHODS

Mosquitoes: The bioassay trials were conducted with *Ae. aegypti* obtained from the London School of Hygiene and Tropical Medicine. The larvae were reared in bowls of tap water and fed on finely ground desiccated liver. Adult mosquitoes were provided with a constant supply of 5% sucrose solution with added honey. The rearing and bioassay rooms were maintained at 27 ± 2°C and 70 ± 10% relative humidity. The rearing room was kept on a 12-h light/12-h dark cycle of 170.0 W/m^2 and 0.3 W/m^2, respectively, with a 30-min dusk period of 0.5 W/m^2 at the day/night interface.

The wind tunnel: The wind tunnel consisted of an aluminium tunnel 0.3 by 0.3 m square and 1.0 m long connected to the bioassay equipment (Figs. 1 and 2). The bioassay equipment was composed of a 0.3-m cubic framework of stainless steel rods that supported 4 identical open-ended glass cylinders that were 4 cm in diameter. The cylinders were connected via glass tubing to a 100-liter heated water tank. Each cylinder was centrally positioned in front of a bioassay chamber. The 4 bioassay chambers were stainless steel frames 0.15 by 0.15 m square and 0.7 m long, covered in thin, 50-μm (200-gauge) transparent Layflat plastic tubing (Polybags Ltd., Lyonway, Greenford, Middlesex, United Kingdom) and sealed at both ends with mosquito netting. A 370-W centrifugal fan drew air into the wind tunnel and passed the air through 2 tightly packed filters containing activated charcoal and a zeolite (5A) molecular sieve, respectively. Filtered air was drawn over the 4 cylinders and through the 4 bioassay chambers and was expelled from the bioassay room by an 18-W centrifugal exhaust fan. The bioassay equipment was kept at atmospheric pressure by balancing the air flow through the 2 fans. Air entering the tunnel was regulated by a mechanical shutter on the inlet fan. A variable resistor controlled the air flow through the exhaust fan. Airspeed through the bioassay equipment was maintained at 0.1 m/sec. The air temperature within the bioassay equipment was the same as the bioassay room temperature, 27 ± 2°C.

Video recording equipment: Four Sanyo CCD VC-2512 video cameras (Sanyo, Osaka, Japan) were situated downwind of the 4 bioassay chambers and focused onto the 4 cylinders. All 4 images were simultaneously recorded as a single image with a superimposed concurrent time signal by use of a Panasonic WJ.450 Quad Splitter (Matsushita Electric, Osaka, Japan) linked to a FOR *A* VTG.22 time–date generator and a video recorder (Fig. 3).

Experimental procedure: Twenty 4- to 7-day-old

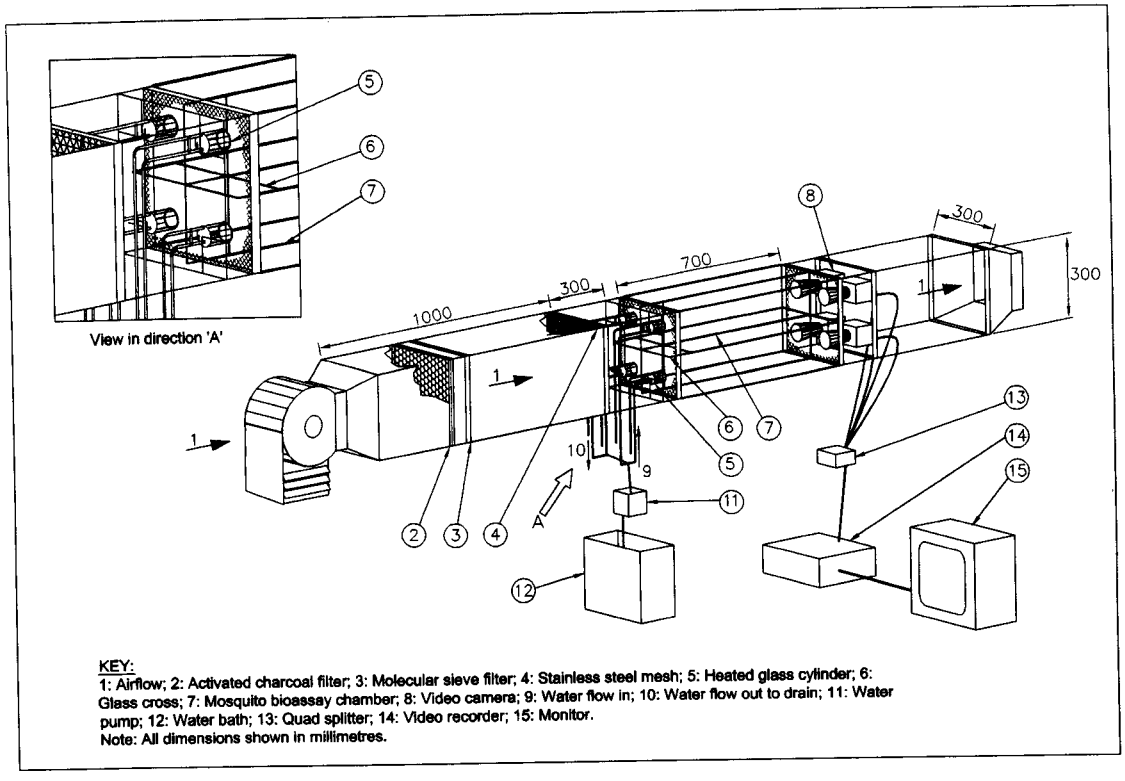

Fig. 2. Schematic diagram of bioassay system.

female *Ae. aegypti* were placed into each bioassay chamber. Prior to testing, the female mosquitoes had been kept in cages containing 400–500 mosquitoes of mixed sex with access to sugar solution. To reduce variation in mosquito activity due to circadian rhythms, all bioassays began at 1200 h.

The open ends of the 4 glass cylinders were covered in 2 layers of cleanly plucked, 3-week-old chick skin. The temperature of the skins was raised to 34°C (human skin temperature) by passing heated water at 300 ml/min through each cylinder. Initial trials with mature chicken skin often leaked water where adult feathers had been removed. However, the double layer of chick skin rarely leaked. To avoid contamination, we did not recycle the water. Temperature variation during the bioassay was minimized (<1°C) by the large capacity of the water tank. Three of the skins were individually treated with 30 µl of ethanol, which was just sufficient to wet the surface area of a skin. The ethanol contained deet (Sigma Chemical Company, Poole, United Kingdom; minimum purity 97%) diluted to give a range of doses (0.32, 0.16, 0.08, 0.04, 0.02, 0.01, 0.005, or 0.0025 mg/cm^2) when applied to a skin. The 4th skin, the control, was untreated.

The 4 bioassay chambers were aligned with the 4 cylinders, and air was passed through the bioassay equipment. To prevent cross contamination occurring between the chambers, a glass cross was inserted at the midpoint of the 4 chambers. The cross protruded upwind, isolating each cylinder.

To activate the mosquitoes in the flight chambers, an experimenter exhaled twice into the inlet duct of the wind tunnel. Human exhalations were known to produce pulselike fluctuations in the concentration of carbon dioxide within the airflow in the wind tunnel. Pulses of carbon dioxide had been observed to activate *Anopheles gambiae* Giles s.s. mosquitoes, which took off and flew upwind (Healy and Copland 1995). The exhalations had the same effect on *Ae. aegypti,* and the mosquitoes in the flight chambers flew upwind toward the skins.

After the exhalations, a video recording of the mosquitoes that landed on the skins was taken for 10 min. The bioassay chambers were then removed, and the skins were left for 1 h at 34°C within the airflow of the wind tunnel. The chambers were then realigned with the cylinders as in the initial bioassay, and the recording procedure was repeated. After the 1-h bioassay, the mosquitoes were killed, and the Layflat tubing, mosquito netting, and skins were discarded. All the stainless steel frames were soaked overnight in 5% decon solution and washed in water. The glass cylinders were soaked overnight in chromic acid, washed in water, soaked in decon, and again washed in water. To prevent contamination of the bioassay equipment with any behaviorally active compounds derived from human skin,

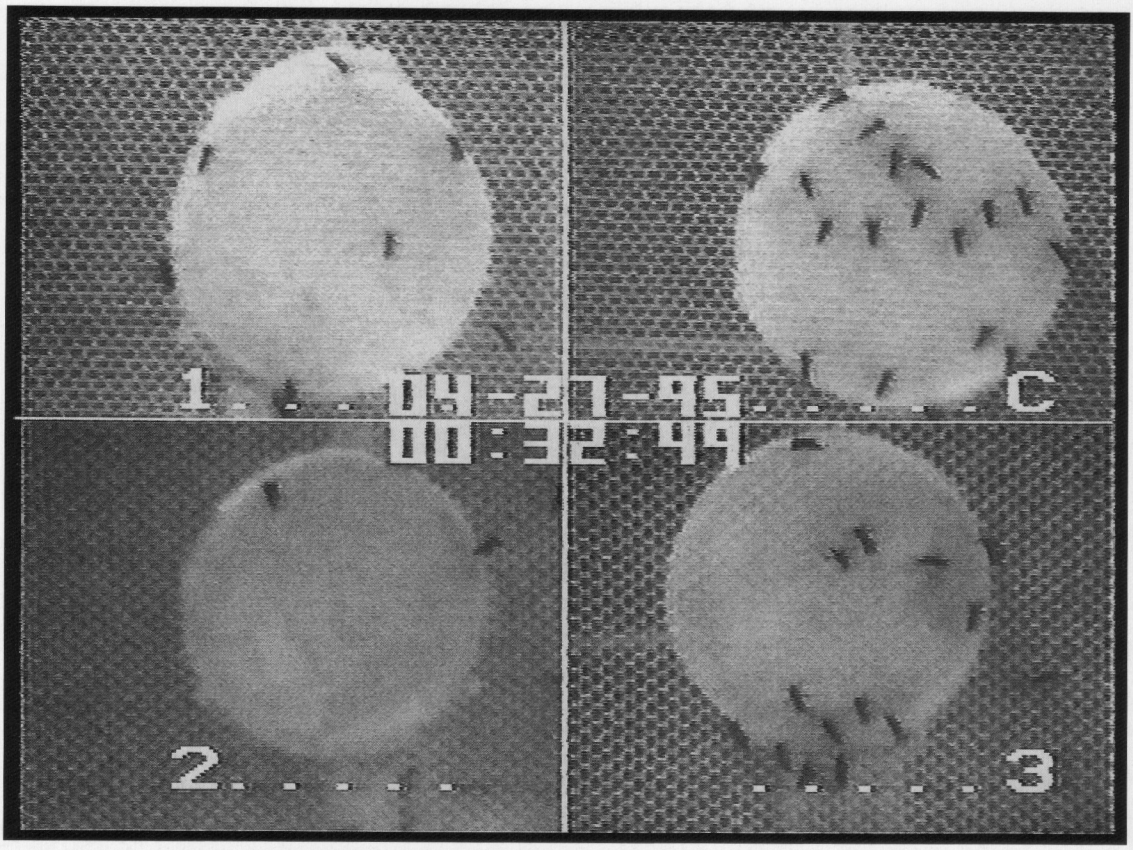

Fig. 3. Video recording of the bioassay in operation. C = untreated control. 1, 2, and 3 = doses of deet.

we wore disposable plastic gloves during assembly and operation of the bioassay.

The range of deet doses was bioassayed and replicated 5 times. The position of the control skin was systematically rotated throughout the series of bioassays. The response of the mosquitoes to chick skins treated with 30 μl of ethanol was recorded. Variation between a set of 4 untreated chick skins was also examined.

Statistical analysis: The number of mosquitoes on each skin was recorded by freeze-framing the video tape at 10-sec intervals throughout both of the 10-min recording periods. Mean values of the number of mosquitoes on the control skin and on the 3 treated skins were calculated for the initial recording period, i.e., immediately after the deet was applied and also for the recording period 1 h after application. The mean value recorded on a treated skin was then calculated as a proportion of the mean value of the corresponding control. The observed reduction in mosquito numbers on a treated skin, in comparison to the control, was assumed to represent the proportion of mosquitoes that had been repelled by the dose of deet. The proportion of mosquitoes repelled was logistically transformed (Crawley 1993) and regressed against $\log_e$ of the dose of deet by generalized linear interactive model (McCullagh and Nelder 1983). The 50% and 90% effective dose (ED50 and ED90) values were calculated, and confidence limits (CL) were estimated by Fieller's theorem (Collet 1991).

RESULTS

The results of the bioassay for the initial and 1-h periods are shown in Figs. 4 and 5, respectively. The results for the initial period show a dose–response relationship between deet and the proportion of mosquitoes repelled. Variation between replicates is comparatively low, indicating the reproducibility of the system. The highest standard error of the mean (SEM) recorded for a dose of deet was ±4.4%. The results for the 1-h bioassay also demonstrate a dose–response relationship, but there is a noticeable increase in variation between replicates of the lower doses. The highest SEM was ±7.6%.

There was no significant difference observed in the response of the mosquitoes to a series of 5 replicates of skins treated with 30 μl of ethanol and untreated controls.

The 4 untreated chick skins gave mean values of 13.38, 12.92, 14.70, and 13.89 (95% CL, 12.51–14.94) during the initial bioassay and mean values

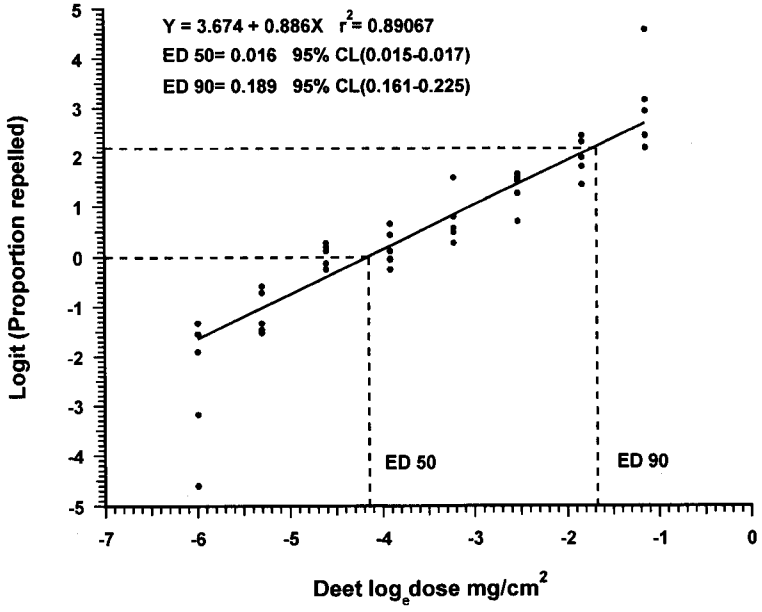

Fig. 4. Proportion of *Ae. aegypti* repelled by deet during initial bioassay.

of 12.55, 12.00, 11.88, and 13.12 (95% CL, 11.48–13.29) during the 1-h bioassay.

DISCUSSION

The wind tunnel design resulted in a high percentage of *Ae. aegypti* responding to the test stimuli. When human breath was introduced to the air flow, the mosquitoes took off, flew upwind, and landed on the upwind netting. Mosquitoes in the control flight chamber rapidly located the skin and probed its surface. Freeze-frame counts typically recorded around 70% of the mosquitoes on the control skin at any one time. There was a continual flux of mosquitoes landing, probing, or leaving the skin. It was considered that, apart from rare individuals, all of the mosquitoes would contact the skin during the bioassay period. At the higher doses

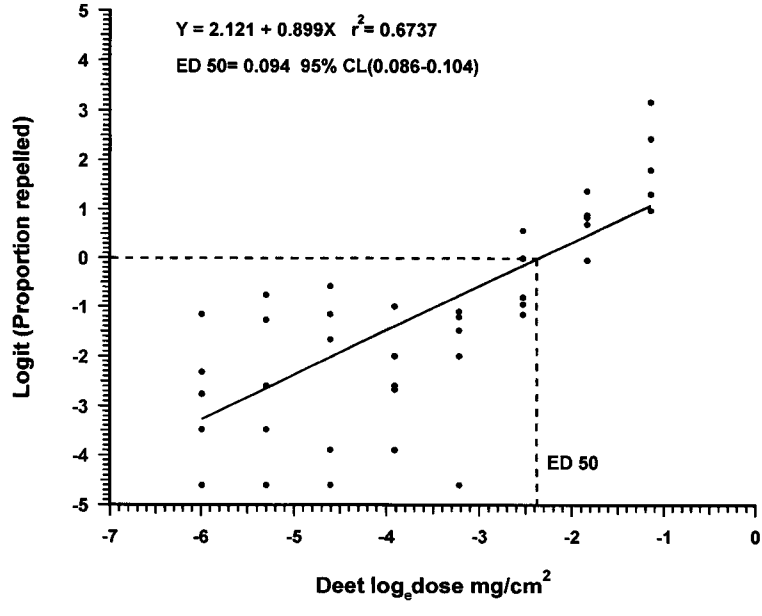

Fig. 5. Proportion of *Ae. aegypti* repelled by deet after 1 h.

Table 1. Effective dose values for deet against *Aedes aegypti*.[1]

Author	Date	Methodology	ED50[2] (mg/cm²)	ED90 (mg/cm²)	ED95 (mg/cm²)
Bar-Zeev and Smith	1959	Independent membrane feeder	0.26	—	—
Rutledge et al.	1976	Free-choice membrane feeder	0.031	0.120	—
Rutledge et al.	1978	Free-choice membrane feeder	0.024–0.042	—	—
Buescher et al.	1982	Free-choice cage on human arm	0.0034	—	0.0145
Rutledge et al.	1983	Free-choice membrane feeder	0.02874	—	—
Buescher et al.	1983	Free-choice cage on human arm	—	—	0.0111
Curtis et al.	1987	Free-choice cage on human arm	0.0016	0.0057	—
Buescher et al.	1987	Free-choice membrane feeder	0.0287	—	0.1301
Gupta et al.	1989	Free-choice cage on human arm	0.0018	—	0.0086
			0.0026		0.0137
Cockcroft et al.	1998	Independent biting test on human arm	0.05 (0.03–0.09)[1]	0.54 (0.20–7.88)	—
		Independent membrane feeder	0.35 (0.17–0.45)	0.79 (0.59–2.26)	—
Present study	2000	Independent wind tunnel bioassay	0.016 (0.015–0.017)	0.189 (0.161–0.225)	—

[1] Values in parentheses are 95% confidence limits.
[2] ED50, 50% effective dose; ED90, 90% effective dose; ED95, 95% effective dose.

of deet, the mosquitoes were observed to approach within a few centimeters of the treated skin and then turn away sharply. Mosquitoes exposed to lower doses landed on the skin but took off after a comparatively short time.

The increase in variation between replicates of the lower doses during the 1-h trial was unexpected. The linear dose–response relationship implies that these low doses should result in almost negligible levels of repellency. However, the results indicate that occasionally a low dose can repel 36% of the mosquitoes.

One possible explanation is that variation in the physical factors that affect the processes of absorption and evaporation that reduce the dose of deet over the hour period may have a more pronounced effect on the lower doses in comparison to the higher doses.

Comparing previously published values of effective doses of deet for *Ae. aegypti* (Table 1) indicates that different techniques produce different values. ED50 values for the free-choice cage, when used on membranes (approximately 0.03 mg/cm²), are typically an order of magnitude lower than independent membrane tests (approximately 0.3 mg/cm²). It is expected that a free-choice design will produce lower values than an independent system because the mosquitoes can move to the lower doses (Rutledge et al. 1976). Curtis et al. (1987) have demonstrated that the apparent effectiveness of a repellent can be influenced by the choice available to the mosquitoes. When the free-choice cage is strapped onto a human arm, the ED50 values (approximately 0.003 mg/cm²) are an order of magnitude lower than the free-choice membrane results. This apparent increase in effectiveness is probably related to the untreated human arm being a more attractive stimulus than a membrane; this results in more mosquitoes biting the control and the low doses.

The wind tunnel bioassay is a system where the doses are tested independently, and chick skin is used as an alternative to human skin. The wind tunnel system also has the effect of activating host-seeking behavior in a high percentage of the test population of mosquitoes. The ED values are approximately equivalent to the free-choice membrane bioassay results. Both of these bioassays give results that are lower than, but in the same order of magnitude as, the independent biting test on a human arm conducted by Cockroft et al. (1998).

The wind tunnel bioassay has been used in commercial studies (patent WO 96/08 147) to screen an extensive range of compounds for repellency. Three compounds are tested simultaneously at the deet ED90 dose, allowing the performance of each compound to be compared against deet. The 1-h trial has proven an important feature, as some compounds can elicit comparable repellency to deet during the initial trial, but very few are comparable after 1 h. Compounds or combinations of compounds that produce effective levels of repellency in both trials are further evaluated by a biting test on a human arm.

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