Repellency of IR3535, KBR3023, *para*-menthane-3,8-diol, and Deet to Black Salt Marsh Mosquitoes (Diptera: Culicidae) in the Everglades National Park

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ABSTRACT IR3535, KBR3023, *para*-menthane-3,8-diol (PMD), and deet were evaluated in controlled studies with human subjects ($n = 5$) for repellency to black salt marsh mosquitoes (*Ochlerotatus taeniorhynchus* Wiedemann), in the Everglades National Park, FL. In tests of 6-h duration, with an average mosquito biting pressure on exposed forearm skin of 19.5 (±13.7) bites per minute, the mean percent repellencies (SE) for IR3535, KBR3023, PMD, and deet was 88.6 (3.2), 97.5 (1.7), 89.2 (2.9), and 94.8 (2.5), respectively. Mean complete protection times (SE) for IR3535, KBR3023, PMD, and deet were 3.0 (1.0), 5.4 (0.6), 3.8 (1.4), and 5.6 (0.5) h, respectively. Untreated (ethanol) controls provided 0% repellency. When mosquito biting rates on the untreated forearm skin of repellent-treated subjects were compared with biting rates on the forearm skin of control subjects, the former were 23%–40% lower early in tests and as much as 22% higher late in tests. These differences cast doubt on the technical merit of test designs comprising evaluation of more than one repellent at a time on the same human subject while underscoring the importance of untreated subjects as negative controls in field repellent bioassays.

KEY WORDS *Ochlerotatus taeniorhynchus* repellent field testing, bioassay

THE INSECT REPELLENT deet (*N*,*N*-diethyl-3-methylbenzamide) was discovered one half century ago (McCabe et al. 1954). Today, 38% of the U.S. population uses a deet-based repellent product and worldwide usage exceeds 200,000,000 applications annually (USEPA 1998). Deet is effective against most biting flies and is sold in aerosol and pump spray formulations and as creams, lotions, solutions, gels, sticks, foams, and towelettes containing from 5%–100% active ingredient (Fradin 1998).

Recent commercialization of a number of plant essential oils as "natural" mosquito repellents (Fradin 1998), and in some cases as purported deet alternatives, is a response to changes in federal regulations that affect minimum risk pesticides (USEPA 1996). But essential oils have limited insect repellent potential; high concentrations are required for repellency, and can cause dermatitis, whereas low concentrations (0.05% to 15% in most 'natural' products) do not repel anthropophilic mosquito species (Barnard 1999). The major concern with natural repellents, however, is the safety of users who may incorrectly assume they are protected from insect bites and infection with arthropod-borne disease agents.

One promising natural product-based repellent is *para*-menthane-3,8-diol (PMD). PMD is from the waste distillate of lemon eucalyptus oil extract (Brady and Curtis 1993). Field tests in Tanzania showed the repellency of PMD was comparable to deet against *Anopheles* mosquitoes (Trigg 1996).

Two promising synthetic repellents are IR3535 (3-[*N*-butyl-*N*-acetyl]-amino propionic acid, ethyl ester) and KBR3023 (1-(1-methyl-propoxycarbonyl)-2-(2-hydroxy-ethyl)-piperidine). Field studies of IR3535 indicate ≥90% repellency for 6 h against *Anopheles* spp. and repellency to *Aedes* and *Culex* spp. (Marchio 1996, Constantini et al. 2000, Thavara et al. 2001). In Malaysia, KBR3023 is repellent to *Aedes* and *Culex* mosquitoes, with effectiveness in some cases exceeding deet (Yap et al. 1998).

In this study, we compared IR3535, KBR3023, and PMD with deet for repellency to *Ochlerotatus taeniorhynchus* Wiedemann in the Everglades National Park, FL. *O. taeniorhynchus* is an important pest of humans and livestock along the south Atlantic and Gulf coasts of the United States and is a vector of Venezuelan equine encephalitis virus (Nayar 1985). For the last four decades, this mosquito has been an important experimental target of research efforts to develop biting fly repellents and personal protection technology (Schreck et al. 1984).

This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use.

Written informed consent was obtained for all human subjects used in this study in accordance with protocol IRB-01 #445-96 as currently approved by the University of Florida, Health Sciences Center, Institutional Review Board for Human Subjects.

Table 1. Mean *BR* (SE) and mean log *BR* for *O. taeniorhynchus* on human subjects, Everglades National Park, FL, 13–15 June 2000

Mean[ab]	Observation period						
	1	2	3	4	5	6	7
Morning[c]							
BR	68.1 (12.3)a	16.9 (7.1)b	22.3 (14.3)b	10.2 (3.3)b	8.3 (2.5)b	8.3 (3.3)b	2.6 (0.3)b
Afternoon[d]							
BR[e]	3.3 (3.2)	2.8 (0.8)	5.3 (2.7)	6.2 (2.2)	4.2 (0.2)	7.3 (4.0)	3.8 (1.2)
Combined[f]							
BR	42.2 (17.2)a	11.3 (5.2)b	15.5 (8.9)b	8.6 (2.2)b	6.7 (1.7)b	7.9 (2.2)b	3.1 (0.6)b
$\log_{10}$ *BR*[e]	1.317	0.874	0.989	0.901	0.793	0.851	0.514

BR, bite rate.
$n = 5$.
[a] Means in each row followed by the same letter are not significantly different ($P < 0.05$, Tukey's honestly significant difference).
[b] Biting rates based on 1-min counts.
[c] 0730 to 1330 h.
[d] 1345 to 1945 h.
[e] Fitted model not significant at $P = 0.05$.
[f] Morning and afternoon data combined by observation period (see text for details).

An additional objective for this study was to evaluate the repellent field test procedure described in ASTM 939-94 (ASTM 2000), which comprises simultaneous testing of two repellents on the same human subject. To do this, we tested the null hypothesis that mosquito biting rates on the untreated skin of repellent treated subjects were no different than biting rates on untreated (control) subjects.

Materials and Methods

Repellent Treatments and Control. Test repellents included: (1) technical deet at 25% in ethanol (EtOH), (2) technical IR3535 at 25% in EtOH, (3) technical KBR3023 at 25% in EtOH, (4) PMD at 40% in a proprietary formulation, and (5) the control, which consisted of 25% deionized water in ethanol. Repellents (1), (2), and (3) were provided by the World Health Organization, Pesticide Evaluation Scheme, Geneva, Switzerland; repellent (4) was provided by the Wisconsin Pharmacal Company, Jackson, WI, USA.

Test Site and Test Methods. Field tests of each repellent were carried out at Snake Bight Trail, near Flamingo, FL, in the Everglades National Park, FL. Five separate tests were made between 13 and 15 June 2000. Each test was 6 h long and ran from 0730 to 1330 hours (morning) or from 1345 to 1945 hours (afternoon). Only the morning test was conducted on 15 June. Before each test, one of the four repellents or a control was randomly assigned each of five (male) human subjects. No subject received the same repellent or the control twice during the study ($n = 5$ for each treatment and the control).

At the commencement of a test, the right or left arm of each subject (selected at random) was treated with an assigned repellent or the control. A dose of the test material was applied to the forearm skin and spread evenly between the wrist and the elbow at the rate of 1 ml/650 cm^2 of skin surface area. The opposite arm received no treatment. Protective gloves, a head net, boots, pants, and a long sleeve shirt (sleeve on treated forearm rolled up throughout test), all worn over regular clothing, were used to standardize clothing color and to prevent mosquito bites on untreated body areas.

Counts of mosquitoes that landed on and probed the forearm skin of the repellent treated subjects (those receiving IR3535, KBR3023, PMD, or deet) were recorded by each subject during a 3-min observation period, the first of which occurred 15 min after application of the test sample (observation period 1) and then again at 1-h intervals for 6 h (observation periods 2–7). Counts of mosquitoes that landed on and probed the forearm skin of the control subject were recorded in the same manner except that the observation period was limited to 1 min to avoid excessive mosquito bites. The order of entry of subjects into the test area was randomized for each observation period and a minimum distance between subjects of 15 m was maintained at all times during testing. At the end of a test, the test sample was washed from the skin using soap and water.

Repellent efficacy was calculated as percentage repellency (%*R*) according to the formula.

$$\%R = ((C - T)/C) \times 100,$$

where C is the total number of mosquitoes biting on the forearm of the control subject in a 1-min observation period, multiplied by 3, and T is the total number of mosquitoes biting on the forearm of a repellent-treated subject in a 3-min observation period.

We used complete protection time (CPT) as a second measure of repellent efficacy. CPT was the time elapsed (in hours) between repellent application and the observation period *immediately* preceding that in which the first mosquito bite on treated skin was observed.

Data Analysis. The biting rate (*BR*) was recorded as the number of mosquitoes that landed on and probed the control subject's (untreated) forearm skin. Because large mean *BR* responses were accompanied by large variances, log transformation was used to minimize heteroscedasticity in *BR* (Steel and Torrie 1980).

Table 2. Mean %*R* (±SE) of deet, IR3535, KBR3023, PMD, and control (EtOH) on human subjects to *O. taeniorhynchus*, Everglades National Park, FL, 13–15 June 2000

Observation period	Mean %*R* (±SE)				
	Deet	IR3535	KBR3023	PMD	EtOH
1	100 (0.0)a[a]	100 (0.0)a	100 (0.0)a	100 (0.0)a	0 (0.0)b
2	100 (0.0)a	97.2 (3.5)a	100 (0.0)a	100 (0.0)a	0 (0.0)b
3	100 (0.0)a	84.8 (8.4)b	100 (0.0)a	95.6 (5.2)ab	0 (0.0)c
4	100 (0.0)a	92.0 (6.5)a	100 (0.0)a	89.4 (7.3)a	0 (0.0)b
5	100 (0.0)a	85.6 (7.2)a	96.2 (5.4)a	96.2 (5.9)a	0 (0.0)b
6	86.4 (8.5)a	79.4 (11.9)a	96.0 (5.8)a	83.2 (6.3)a	0 (0.0)b
7	77.0 (14.2)a	81.0 (14.1)a	90.0 (10.1)a	60.0 (3.9)a	0 (0.0)b
Average	94.8 (2.5)ab	88.6 (3.2)b	97.5 (1.7)a	89.2 (2.9)b	0 (0.0)c

$n = 5$.
PMD concentration is 40% in a proprietary formulation; deet, IR3535 and KBR3023 concentrations each 25% in ethanol.
[a] Means in each row followed by the same letter are not significantly different ($P < 0.05$, Tukey's honestly significant difference test).

Thus, before the calculation of %*R*, and before statistical analysis of *BR* or %*R*, each *BR* datum was transformed to $\log_{10}(BR + 1)$.

The hypothesis of no difference in mean *BR* and mean log *BR* among all observation periods (*OP*), and for *BR* data from morning and afternoon tests combined by *OP*, was analyzed using the model: mean *BR* (or mean log *BR*) = *OP*. The hypothesis of no difference in mean log *BR* on the untreated skin of repellent-treated subjects (BR_t) compared with the untreated skin on untreated subjects (BR_{ut}), was analyzed using the model: $BR_t = BR_{ut}$. For the latter test, measurements of *BR* were made in observation periods 1, 3, 5, and 7 only.

For all tests, raw and log-transformed data were analyzed using analysis of variance (ANOVA) procedures with means separation via Tukey's studentized range honestly significant difference (HSD) test (SAS Institute 1998).

Results

Mosquito Biting Rates. In the morning tests, mean *BR* (on control subjects only) was significantly higher ($F = 8.03$, df = 6,14, $P < 0.001$) than at other times (Table 1). Mean *BR* on control subjects ranged from 68.1 bites per min (morning observation period 1) to 2.6 bites per min (morning observation period 7). Average *BR* for control subjects was 19.5 ± 13.7 bites per min. When morning and afternoon data were combined by observation period, mean *BR* was highest in period 1 ($F = 2.92$, df = 6,28, $P = 0.024$). Mean log *BR* (for *BR* subject to log transformation) did not differ significantly by observation period ($F = 1.83$, df = 6,28, $P = 0.1292$).

Percent Repellency. Ethanol controls provided 0% repellency (Table 2). Deet, IR3535, KBR3023, and PMD provided ≥60% repellency at all times and provided 100% repellency for 4, <1, 3, and 1 h, respectively. Mean log %*R* was highest for KBR3023 and deet. KBR3023 provided significantly higher mean log repellency ($F = 250.1$, df = 4,170, $P = 0.0001$) than PMD or IR3535.

Complete Protection Time. The order of CPT was: deet > KBR3023 > PMD > IR3535 > EtOH (Table 3). Deet and KBR3023 provided 2.6 and 2.4 h more average protection time, respectively, than IR3535 ($F = 9.04$, df = 4,20, $P = 0.0002$), although differences in CPT between deet and KBR3023 (0.2 h), deet and PMD (1.8 h), KBR3023 and PMD (1.6 h), and IR3535 and PMD (0.4 h) were not significant.

Comparisons of *BR* on Repellent-Treated and Untreated Subjects. The presence of repellent on a subject's forearm influenced biting rates on the adjacent untreated forearm of the same subject (Table 4). Differences between BR_t and BR_{ut}, for subjects treated with KBR3023, IR3535, and PMD, although not significant at $P = 0.05$, decreased with observation period. In some cases (IR3535 in observation period 5 and KBR3023 and PMD in observation period 7), BR_t exceeded BR_{ut}. For deet, BR_{ut} exceeded BR_t in all observation periods.

Table 3. Mean complete protection time (SE) in h from bites of *O. taeniorhynchus* on human subjects provided by deet, KBR3023, PMD, IR3535, and control (EtOH), Everglades National Park, FL, 13–15 June 2000

Mean complete protection time				
Deet[a]	KBR3023	PMD	IR3535	EtOH
5.6 (0.5)a	5.4 (0.6)a	3.8 (1.4)ab	3.0 (1.0)b	0 (0.0)c

$n = 5$.
PMD concentration is 40% in a proprietary formulation; deet, IR3535, and KBR3023 concentrations each 25% in ethanol.
[a] Means followed by the same letter are not significantly different ($P < 0.05$, Tukey's honestly significant difference).

Table 4. Mean log *BR* for *O. taeniorhynchus* on control (EtOH) human subjects (BR_{ut}) and on untreated forearm of subjects (BR_t) treated with deet, IR3535, KBR3023, or PMD, Everglades National Park, FL, 13–15 June 2000

Observation period	Mean log BR_{ut} (EtOH)	Mean logBR_t when this repellent on opposite forearm:			
		KBR3023	IR3535	PMD	Deet
1[a]	1.795	1.319	1.381	1.091	1.162
3[a]	1.467	1.211	1.158	1.350	1.270
5[a]	1.270	1.232	1.301	1.126	1.079
7[a]	0.992	1.023	0.967	1.207	0.860
Avg:	1.381	1.196	1.202	1.194	1.093

BR, bite rate.
$n = 100$.
[a] Fitted model not significant at $P = 0.05$.

Discussion

Repellent Efficacy. KBR3023 provided the highest %*R* (97.5), followed by deet (94.8), and was more repellent to *O. taeniorhynchus* than IR3535 (88.6) or PMD (89.2). PMD provided >80% repellency for 5 h, although %*R* for both PMD and deet decreased relatively quickly late in tests. IR3535 was effective, but variably repellent, with %*R* ranging from 84.8 (observation period 2), to 92 (observation period 3), to 79.4 (observation period 6).

The CPT from mosquito bites was longest for deet and KBR3023. Differences in mean CPT between PMD and deet or KBR3023 and between IR3535 and PMD were ≈1.5 and 2.5 h, respectively. All three nondeet repellents protected against mosquito bites and are effective insect repellents for human use. None of the repellents (including deet) caused skin-warming or dermatitis.

The comparison of ethanol-formulated deet, KBR3023, and IR3535 with a proprietary formulation of PMD may have provided some advantage to the latter in these tests. The microencapsulation of other repellents, such as deet, for example, significantly extends CPT (Schreck et al. 1984). Logically, one would expect a similar enhancement of repellency for KBR3023 and IR3535 if formulated for extended release or otherwise to improve activity.

Comments Regarding Repellent Test Methodologies. ASTM E939-94 (ASTM 2000) prescribes the side-by-side comparison of repellents on the same test subject. This technique may not be valid because the presence of repellent on one arm of a subject affects the mosquito biting rate on the opposite (untreated) arm (BR_t) of the same subject. Relative to BR_{ut} (the biting rate on untreated subjects), this difference is manifest as low BR_t early in tests and high BR_t late in tests (Fig. 1). Data for BR_t for PMD in observation periods 3 and 7 (Table 4) help illustrate this relationship: mean log BR_t differs from mean log BR_{ut} by −8% and +22%, respectively; actual %*R* (based on the biting rate on untreated subjects) is 95.6 and 60.0%, respectively. Apparent %*R* (based on the biting rate on repellent treated subjects), however, is 85 and 79%, respectively. Thus, in observation period 3, actual %*R* for PMD is underestimated by 11% but is overestimated in period 7 by 19%.

Although these differences are not statistically significant (at $P = 0.05$), there are biological and technical consequences for ignoring the factors that cause them, including confounding of (repellent) treatment effects, biased estimates of mosquito biting rate, and faulty estimation of %*R*. For these reasons, we recommend a two-fold modification of the repellent testing technique currently described in ASTM 939-94 comprising the evaluation of only one repellent at a time on a human subject and the mandatory use of a valid negative control (human subject without repellent on any part of their body) to estimate mosquito biting rate(s) and to calculate %*R*.

One other concern in field bioassays of repellents is the variance of estimates of mean mosquito biting rate.

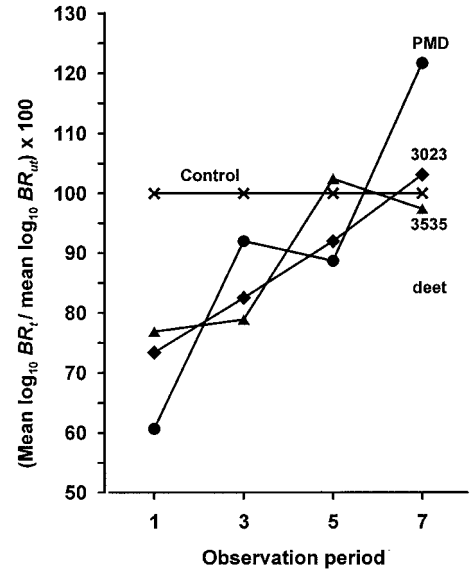

Fig. 1. *Ochlerotatus taeniorhynchus* biting rates on the untreated forearm of human subjects whose opposite forearm has been treated with deet, KBR3023, IR3535, or PMD (mean $\log_{10} BR_t$), calculated as a percent of the biting rate on untreated (control) subjects (mean $\log_{10} BR_{ut}$).

Typically, the innate attractiveness of human subjects to mosquitoes ranges from 30% to 70% (Schreck et al. 1990), thus, estimates of *BR* can be imprecise, particularly when based on small sample size. Increasing the numbers of test subjects improves precision but the resources required to do so quickly exceed practical limits. For example, a 50% improvement in estimated mean BR_{ut} in the current study would require $n = 63$ biting rate observations *in each period* (Steel and Torrie 1980); a 75% improvement would require $n = 251$ observations in each period. As an alternative to large sample sizes, we suggest that test subjects be selected according to their comparative attractiveness to mosquitoes. This factor can be determined using an olfactometer (Posey et al. 1998, Mauer and Rowley 1999), or by other means. Subjects selected for repellent testing would be those with an attractivenes index within ± 1 or 2 SD (depending on the needs of the experiment) of the mean index for mosquito attractiveness for the test population.

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