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Repellent activity of DEET against *Amblyomma cajennense* (Acari: Ixodidae) nymphs submitted to different laboratory bioassays

Atividade repelente do DEET contra ninfas de *Amblyomma cajennense* (Acari: Ixodidae) em bioensaio laboratorial

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Abstract

This study was developed to evaluate the repellent activity of N,N-diethyl-3-methylbenzamide (DEET) against *Amblyomma cajennense* nymphs. Two repellent bioassays were compared and the effective concentration and repellent time were calculated. The fingertip test was accomplished to evaluate in vivo four concentrations of the compound (0.200; 0.100; 0.050 and 0.025 mg.cm^{-2}) and the filter-paper bioassay to evaluate in vitro the two highest concentrations. The compound provided repellence higher than 90% in all concentrations and at least 95% repellency in the highest concentration over 5 hours. The effective concentration against 50% of tested nymphs (EC50) was 0.006 mg.cm^{-2} and the EC99 was 0.036 mg.cm^{-2}. Those concentrations were lower than the ones obtained against other tick species, denoting the effectiveness of DEET against *A. cajennense*. The repellency time against 50% of the ticks (RT50) was 4.8 hours and the RT90 was 2.7 hours. Both bioassays were adequate to evaluate *A. cajennense* repellency and provided similar results; however the in vivo test is more appropriate to estimate the effective concentration and repellency time.

Keywords: DEET, *Amblyomma cajennense*, repellency time, fingertip bioassay.

Resumo

Este estudo foi conduzido com o objetivo de avaliar a atividade repelente do N,N-diethyl-3-methylbenzamide (DEET) sobre ninfas de *Amblyomma cajennense*. Dois bioensaios para a avaliação de repelência foram comparados e cálculos da concentração eficaz e do tempo de repelência foram realizados. Foram empregados o bioensaio da ponta do dedo, para avaliação in vivo de quatro concentações do químico (0,200; 0,100; 0,050 e 0,025 mg.cm^{-2}) e o bioensaio do papel filtro, para a avaliação in vitro das duas concentrações mais altas. O composto conferiu mais de 90% de repelência em todas as concentrações utilizadas e 95% de repelência por mais de cinco horas na maior concentração. A concentração do composto efetiva contra 50% das ninfas testadas (CE50) foi de 0,006 mg.cm^{-2} e a CE99 foi de 0,036 mg.cm^{-2}. Estas concentrações são mais baixas do que as observadas em outras espécies de carrapatos, denotando a efetividade do princípio contra *A. cajennense*. O tempo de repelência de 50% dos carrapatos (TR50) foi de 4,8 horas e o TR90 de 2,7 horas. Os dois bioensaios avaliados permitiram a observação de percentuais de repelência igualmente altos e se mostraram adequados para tal avaliação, sendo que o teste in vivo é mais indicado para cálculo da concentração eficaz e da duração da repelência.

Palavras-chave: DEET, *Amblyomma cajennense*, tempo de repelência, bioensaio ponta do dedo.

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Introduction

Amblyomma cajennense (Fabricius), the Cayenne tick, is a three-host species that, despite the adult's preference for equids, can parasitize other mammals such as bovids, cervids, and wild and domestic canids, as well as birds and human beings (BARROS- BATTESTI et al., 2006). It is spread over the American continent, from the Southern USA to Northern Argentina. Due to its unspecificity it is involved in the transmission of pathogens between animals and humans. It is the vector of *Rickettsia rickettsii* (Wolbach) Brumpt, causal agent of the Rocky Mountain Spotted Fever in Central and South America (DIAS; MARTINS, 1939; BUSTAMANTE et al., 1946; DE RODANICHE, 1953; GUEDES et al., 2005). It is also able to experimentally transmit *Rickettsia parkeri* Lackman et al. (SANGIONI et al., 2005), agent associated with clinical symptoms of a rickettsial fever (PADDOCK et al., 2004) and can be involved in the transmission of other rickettsial agents (BILLINGS et al., 1998).

The control of this tick is difficult due to the large variety of hosts and to the short duration of parasitic phases (LABRUNA et al., 2004). Only commercial pyrethroid formulations are recommended to treat equids. However, field and laboratory assays showed that *A. cajennense* adults are naturally resistant to this base (BITTENCOURT et al., 1987, 1989). In this context, repellents allow a different approach, whose aim is to prevent tick attachment to the hosts, reducing the use of acaricides. The World Health Organization (WHO) recommends the use of personal protection mechanisms in endemic regions against arthropod-borne diseases (BARNARD, 2000).

The compound N,N-diethyl-3-methylbenzamide, previously named N,N-diethyl-m-toluamide and popularly known as DEET (Sigma Aldrich - Sigma-Aldrich 3050 Spruce St. St. Louis, MO 63103), was patented in the Department of Agriculture by the US Army in 1946 and registered for public use in 1957 (FRADIN, 1998). It is a well-characterized compound, recognized as a reference repellent, and it has been extensively used by civilian and military personnel. Annually, over 200 million people use it for individual protection. Through the years, it has shown itself to have an outstanding safety profile and it has not been dangerous to human health nor to the environment (FRADIN, 1998). However, the National Health Surveillance Agency (Anvisa, Brazil) does not recommend it for children under two years old (ANVISA, 2006). It is a large-spectrum repellent, acting against mosquitoes, hematophagous flies, lice, fleas and ticks. Due to its outstanding role as a repellent, it has been tested against different tick species, showing activity against *Amblyomma americanum* Linnaeus, *Ixodes ricinus* Linnaeus, *Amblyomma hebraeum* Koch and *Ixodes scapularis* Say (SCHRECK et al., 1995; STAUB et al., 2002; PRETORIUS et al., 2003; CARROLL et al., 2005).

This study was developed to evaluate the repellent activity of N,N-diethyl-3-methylbenzamide (DEET) against *A. cajennense* nymphs. Two repellent bioassays were compared and the effective concentration and repellency time were calculated, proving its efficacy against *A. cajennense*.

Materials and Methods

1. Ticks and volunteers

A. cajennense engorged females were obtained in naturally infested equines and incubated in a chamber (27 °C, 80% RH) during the oviposition period. Five-day-old larvae were placed on rabbits to feed (*Oryctolagus cuniculus*) using a feeding chamber (SONENSHINE, 1991). Engorged larvae were collected from the rabbits and incubated in the same conditions mentioned above. Unfed nymphs with ages varying from two weeks to two months were tested in the repellency bioassays. The bioassays were carried out on three female volunteers.

2. Repellent bioassays

DEET (Fluka, C12H17NO, MM 191.28, 95% pure, lot 11706212) was purchased from Sigma-Aldrich. Stock solution (7.2% ≈ 0.200 $mg.cm^{-2}$) was prepared using 95% ethanol as the solvent. This concentration is close to those observed in low concentrations of DEET available in commercial formulations. The following concentrations: 0.200, 0.100, 0.050 and 0.025 $mg.cm^{-2}$ diluted in ethanol 95% were used in fingertip bioassays and the two highest in filter-paper bioassays.

3. Fingertip bioassay

This bioassay was developed according to Schreck et al. (1995). The proximal phalange of the left index finger of one volunteer was treated with DEET and the right one with ethanol 95% as the negative control. A volume of 2.75 $\mu L.cm^{-2}$ per treated area was used. The test was performed 10 minutes after the solution had been applied, for solvent evaporation. A nymph was released, individually, on the distal phalange, then the finger was vertically positioned with tip downward, allowing the tick to climb the finger because of its negative geotropism. The ticks that dropped off the finger, inverted their direction after touching the treated area, or remained on the release point after 1 minute were considered repelled. To evaluate the repellent time, the tests were repeated after 50 minutes and later every hour, until repellence was lower than 50%. Thirty ticks were evaluated for each concentration. All nymphs were previously tested in the negative control and only the active ones were tested with DEET. One hour after the first treatment interval (10 minutes) the mortality of the tested nymphs was evaluated. To evaluate behavior alterations, the surviving nymphs were submitted to fingertip bioassay again, using DEET in the same concentration as the previous test.

4. Filter-paper bioassay

This bioassay was developed according to Carroll et al. (2004). A piece of filter paper (10 cm long × 6 cm wide) was divided into three strips of 2.2, 3.3 and 4.5 cm long by 6 cm wide. The middle strip (3.3 × 6 cm) was treated with 165 µL of DEET or ethanol 95% as the negative control. The filter paper was

vertically positioned and attached in the middle of a Petri dish (9 cm diameter). Five ymphs were released on the lower strip and their movements were evaluated during 5 minutes. The evaluation of repellency and the repellency time were estimated similarly as mentioned above in the fingertip bioassay. Ten repetitions were done for each concentration. All nymphs were previously tested in the negative control and only the active ones were tested with DEET.

5. Statistical analysis

The comparisons among the concentrations and the bioassays were done by the chi-square test using a significance level of $p < 0.05$. When the percentage of repellency was significantly higher than in the control, that concentration was considered as repellent. The estimate of effective concentrations (EC) and repellency time (RT) against 50, 90, 95 and 99% of tested nymphs (EC50, EC90, EC95, EC99 and RT50, RT90, RT95 and RT99) were done by probit analysis (Priprobit Copyright – C 1996-2000. Masayuki Sakumo – All rights reserved. Ver 1.63), using the results obtained in fingertip bioassay.

Results

1. Fingertip bioassay

A. cajennense nymphs were repelled in all tested concentrations. Even in the lowest concentration high percentages of repellency were obtained; however, after one hour of observation, there was a significant decrease in repellency. At the 0.200 and 0.100 mg.cm^{-2} concentrations, the first repellency rates were 100%, and they were near 97% up to 4 hours in the higher concentration. Even after 5 hours, the observed repellency observed was significantly different from the ethanolic control at 0.200 mg.cm^{-2}. The reduction of DEET concentration interfered mainly in the repellency duration (Table 1).

The EC50, EC90, EC95 and EC99 in the first hour were 0.006, 0.025, 0.036 and 0.075 mg.cm^{-2}, respectively. The RT50 at 0.200 mg.cm^{-2} concentration was nearly 5 hours and the RT90 in the same concentration was 1 hour 41 minutes. The RTs decreased with the reduction of the concentration (Figure 1) and at 0.025 mg.cm^{-2} the RT99 was 0.1 hour (= 6 minutes).

Nearly 30% of the ticks tested in the two highest concentrations died 1 hour after the test. All ticks which came in contact with the finger-treated area, even the ones that were not repelled, exhibited behavior alterations, such as locomotion with large and slow steps trying to keep their bodies away from the repellent.

2. Comparison between bioassays

Significant difference between the tests was observed only after 5 hours, in the 0.200 mg.cm^{-2} concentration, with rates of 100 and 33% in filter-paper and fingertip, respectively. In this concentration, in the filter-paper test an expressive decrease in the repellence rate was observed only 20 hours after the beginning of the test, reaching 36% of repellence. In 0.100 mg.cm^{-2} concentration, a statistical difference between the tests was observed 3 hours after the beginning of the test. Higher rates of repellence were observed in filter-paper (80%) than in fingertip bioassay (43%) (Table 2).

Table 1. Duration and percentage of repellency of four concentrations of DEET against *Amblyomma cajennense* nymphs, using fingertip bioassay.

Conc. mg.cm^{-2}	Repellency %					
	10 minutes	1 hour	2 hours	3 hours	4 hours	5 hours
0.200	100 $^{a, A}$	97 $^{a, A}$	93 $^{a, A}$	100 $^{a, A}$	97 $^{a, A}$	33 $^{a, B}$
0.100	100 $^{a, A}$	93 $^{a, A}$	67 $^{b, B}$	43 $^{b, B}$	-	-
0.050	97 $^{a, A}$	67 $^{b, B}$	0 $^{c, C}$	-	-	-
0.025	90 $^{a, A}$	40 $^{c, B}$	0 $^{c, C}$	-	-	-
Control	0 ^{b}	0 ^{d}	0 ^{c}	0 ^{c}	0 ^{b}	0 ^{b}

Different lower-case letters between concentrations and capital letters between repellency duration indicate significant difference by chi-square test ($p < 0.05$).

- These tests were not done because repellence at previous concentration was lower than 50%

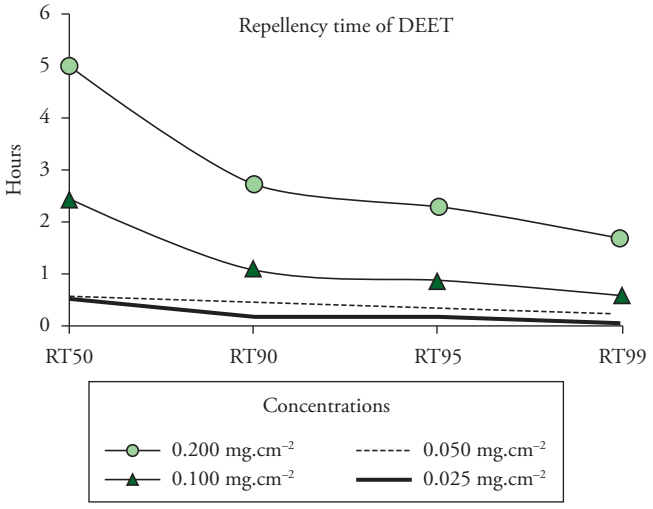

Figure 1. Repellency time of 50, 90, 95 and 99% (RT50, RT90, RT95 and RT99) of *Amblyomma cajennense* nymphs tested in different concentrations of DEET in a fingertip bioassay.

Discussion

In the present study *A. cajennense* nymphs were greatly repelled by DEET in all tested concentrations, and more than 90% of protection was observed during the 10 minutes-1 hour interval. The 0.200 mg.cm^{-2} concentration, equivalent to a 7.2% solution, guaranteed a 95% repellency over a 4 hours period. In some species such as *A. americanum* (SCHRECK et al., 1995; SOLBERG et al., 1995) and *Ixodes scapularis* (CARROLL et al., 2005, 2007) repellency rates similar to those obtained in the present study were observed only in higher concentrations. On the

Table 2. Duration and percentage of repellency of two DEET concentrations (mg.cm^{-2}) against *Amblyomma cajennense* nymphs using filter-paper and fingertip bioassays.

Duration	Fingertip		Filter-paper		Control
	0.200	0.100	0.200	0.100	
10 minutes	100 $^{a, A}$	100 $^{a, A}$	100 $^{a, A}$	100 $^{a, A}$	0 ^{B}
1 hour	97 $^{a, A}$	93 $^{a, A, B}$	98 $^{a, A}$	84 $^{b, B}$	0 ^{C}
2 hours	93 $^{a, A}$	67 $^{b, B}$	98 $^{a, A}$	70 $^{b, B}$	0 ^{C}
3 hours	100 $^{a, A}$	43 $^{b, C}$	98 $^{a, A}$	80 $^{b, B}$	0 ^{D}
4 hours	97 $^{a, A}$	-	100 $^{a, A}$	-	0 ^{B}
5 hours	33 $^{b, B}$	-	100 $^{a, A}$	-	0 ^{C}
6 hours			100 $^{a, A}$		0 ^{B}
20 hours	-	-	36 $^{b, A}$	-	0 ^{B}

Different lower-case letters between repellency duration and capital letters between concentrations indicate significant difference by chi-square test ($p < 0.05$).

- These tests were not done because repellency at previous concentration was lower than 50%

other hand, protections of similar magnitude were not obtained against *I. ricinus* (STAUB et al., 2002) in field tests or *A. hebraeum* (PRETORIUS et al., 2003) in fingertip bioassay, even using higher concentrations. Salafsky et al. (2000) observed only partial repellency of *A. americanum* adults using commercial 20% DEET formulations and no protection against *Dermacentor variabilis* Say. The compound did not repel *Amblyomma variegatum* Fabricius adults when released with an attractant stimulus (MCMAHON et al., 2003). Based on the results of the present study and on the literature it is clear that *A. cajennense* is more sensitive to DEET than other tick species so far evaluated.

The ECs values observed in the present study reinforce the aforementioned results regarding the high sensitivity of *A. cajennense* to the compound, considering that they were lower than those obtained by Carroll et al. (2004) to *I. scapularis* and *A. americanum*, using filter-paper bioassay. When using the fingertip bioassay, Carroll et al. (2007) found ECs to *I. scapularis* close to the present study and did not find any repellent activity against *A. americanum.*

It was observed that the increase of DEET concentration did not interfere with the initial repellency rate, but increased significantly the repellent time. Similar results were observed by Carroll et al. (2005), testing DEET against *I. scapularis* and *A. americanum* in relation to the initial repellency rate, and by Fradin and Day (2002) when testing DEET against the mosquito *Aedes aegypti* Linnaeus in relation to repellency time.

The largest increase in repellency duration was observed when the concentration was augmented from 0.100 to 0.200 mg.cm^{-2}; lengthening the protection from 1 to 4 hours, RT90 similar to that obtained here, 2.7 hours at 0.200 mg.cm^{-2} concentration, was observed by Schreck et al. (1995) in *A. americanum*, although using a higher concentration of 0.300 mg.cm^{-2}. Against *A. hebraeum,* lower RTs were observed by Pretorius et al. (2003), who obtained 2 hours protection using a 20% DEET commercial formulation, and by Jensenius et al. (2005), who verified protection against 90% of ticks for a interval lower than 1h using DEET in concentrations as high as 80%. While Fradin and Day (2002) observed more than 5 hours of total protection using a 23.8% formulation against *A. aegypti,* Chou et al. (1997) found high repellency efficacy for 8 hours utilizing formulations with 95% of DEET. Mathematic models of efficacy and persistence of repellents in mosquitoes show that the protection conferred by DEET is proportional to the logarithm of its concentration, with the higher concentrations promoting longer protection (RUTLEDGE et al., 1985). However, this curve reaches a plateau at the 50% concentration, and additional protection supplied is lower with each increase of the dose (BUESCHER et al., 1982). The highest concentration used here is lower than those used in the aforementioned studies and even lower than the plateau set up by Buescher et al. (1982). The American Association of Pediatrics recommends the use of DEET between 10 and 30% concentration for children (AAP COMMITTEE ON ENVIRONMENTAL HEALTH, 2003). This dose can guarantee lasting protection avoiding intoxication, as children are more sensitive to DEET. Therefore there is a potential use of DEET to protect against *A. cajennense*, as it is possible to increase the concentration and, consequently, the repellency duration, with no hazard to human health.

The neurotoxic effects such as locomotor alterations and death after exposure to DEET were described for other arthropods (LICCIARDI et al., 2006), but as far as we know, they are reported for ticks for the first time. Salafsky et al. (2000) observed high mortality of *A. americanum* and *D. variabilis* when exposed to LIPODEET, a long-lasting action formulation of DEET, but it was not proved for DEET.

Similar high rates of repellency were observed in both bioassays. However, nymphs were repelled for a longer time in filter-paper than in fingertip bioassay. Those results are easily explained, as fingertip bioassay is a more rigorous test than filter-paper, because the ticks are more motivated to climb an attractive substrate such as a finger than a piece of filter-paper. Due to the low motivation to climb a piece of paper, this kind of bioassay has the disadvantage of not filtering weak repellents (DAUTEL, 2004). The host skin possesses characteristics that interfere with the efficacy and duration of the repellent activity of a compound; these include: high temperature, transpiration, skin absorption and substances that can combine with repellent molecules. Those factors can contribute to a faster loss of repellency in compounds tested on skin than on paper (MAIBACH et al., 1974).

Based on the results, it is possible to conclude that DEET is an effective repellent against *A. cajennense* nymphs and can be adopted as a reference for future evaluations of compounds for candidates as repellents and for individual protection. Both bioassays, fingertip and filter-paper, are suitable for evaluation of repellency in this species, as the behavior pattern is similar in both. However, for effective concentration and repellence duration estimations, fingertip is more appropriate, as it is a more rigorous test and consequently closer to reality.

DEET has a remarkable safety profile after 40 years of use, and DEET-based repellents remain the gold standard of human protection under circumstances in which it is crucial to be protected against arthropod bites that might transmit disease (FRADIN, 1998). The observation of repellent efficacy of this compound at low concentrations against *A. cajennense* nymphs allows its use as

part of a strategy to prevent spotted fever in Central and South America, where *A. cajennense* is the main vector of *R. rickettsii.*

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Commitee of Ethics

The use of the animals and the participation of human beings were approved by the Research Ethics Committee from UFG (protocol nº11 05/03/2007). The studies with animals were carried out in accordance with ethical norms.

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