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Klafke, Guilherme; Costa Fiorini, Leonardo; Medeiros de Barros, Antônio Thadeu
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Characterization of the pyrethroid resistance profile of *Rhipicephalus (Boophilus) microplus* populations from the states of Rio Grande do Sul and Mato Grosso do Sul, Brazil

Caracterização do perfil de resistência do *Rhipicephalus (Boophilus) microplus* a piretróides em populações dos estados do Rio Grande do Sul e Mato Grosso do Sul, Brasil

Márcia Cristina Mendes^{1*}; Fernanda Calvo Duarte¹; João Ricardo Martins²; Guilherme Marcondes Klafke²; Leonardo Costa Fiorini¹; Antônio Thadeu Medeiros de Barros³

¹Instituto Biológico, São Paulo, SP, Brasil

²Laboratório de Parasitologia, Instituto de Pesquisas Veterinárias “Desidério Finamor”,
Fundação Estadual de Pesquisa Agropecuária – Fepagro, Eldorado do Sul, RS, Brasil

³Empresa Brasileira de Pesquisa Agropecuária – Embrapa Pantanal, Corumbá, MS, Brasil

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Abstract

Cattle ticks *Rhipicephalus (Boophilus) microplus* are mainly controlled in Brazil by means of acaricide products, without any official policies in this regard. Acaricides continue to be sold indiscriminately, and this has contributed towards making the problem of resistance widespread, thus making diagnosis and monitoring of tick resistance essential. Here, bioassays (larval packet test) were performed on tick populations from the states of Rio Grande do Sul and Mato Grosso do Sul regarding their susceptibility to cypermethrin, deltamethrin and flumethrin. All the tick samples tested showed resistance to cypermethrin (10) (resistance factor (RF) ranging from 5.6 to 80.3) and deltamethrin (10) (RF ranging from 2.4 to 83.1). Six out of eight populations were resistant to flumethrin (RF ranging from 3.8 to 8.2). PCR molecular analyses did not show any T2134A mutations in the voltage-gated sodium channel gene, in any of the sampled populations. The results from this study highlight the critical status of resistance of the cattle tick to synthetic pyrethroids in the regions studied. Further studies are needed to identify the mechanisms responsible for the resistant phenotypes observed in the bioassays. This was the first detection of flumethrin resistance in Brazil.

Keywords: Cattle tick, acaricide resistance, synthetic pyrethroids, PCR.

Resumo

O controle do carrapato *Rhipicephalus (Boophilus) microplus* no Brasil é feito principalmente com produtos acaricidas, sem uma política de controle oficial. A venda destes produtos continua ocorrendo de maneira indiscriminada, o que tem contribuído para generalizar o problema da resistência em todo o país, tornando essencial seu diagnóstico e monitoramento. Bioensaios (teste do pacote de larvas) foram conduzidos com populações de carrapatos do Rio Grande do Sul e Mato Grosso do Sul, com relação à sua suscetibilidade a três princípios ativos piretróides. Todas as amostras analisadas mostraram resistência à cipermetrina (10), com fatores de resistência (FR) de 5,6 a 80,3, e à deltametrina, com FR variando de 2,4 a 83,1. Seis das oito amostras foram resistentes à flumetrina (FR de 3,8 a 8,2). Análises moleculares utilizando PCR demonstraram que em todas as populações amostradas não foram encontrados alelos com a mutação T2134A no gene do canal de sódio controlado por voltagem. Os resultados deste estudo evidenciam a situação crítica da resistência do carrapato bovino ao grupo dos piretróides sintéticos nas regiões estudadas. Novos estudos são necessários para identificar os mecanismos responsáveis pelos fenótipos resistentes observados nos bioensaios. Este é o primeiro relato da resistência a flumetrina no Brasil.

Palavras-chave: Carrapatos-dos-bovinos, resistência acaricida, piretróides sintéticos, PCR.

*Corresponding author: Márcia Cristina Mendes
Instituto Biológico, CEP 04014-002, São Paulo, SP, Brasil
e-mail: mendes@biologico.sp.gov.br

Introduction

Animal health standards and requirements are at the center of current national and international debates because of ever-increasing consumer consciousness regarding the risks associated with consumption of livestock-derived products. In addition, producers are also faced with concerns regarding environmentally responsible sustainable development.

Controlling cattle ticks *Rhipicephalus* (*Boophilus*) *microplus* is an important challenge for Brazilian cattle producers, given their widespread distribution and the economic losses involved in acquisition and application of acaricides with the aim of reducing tick infection. Furthermore, pesticide residues tend to increase in animal-derived products, since resistant ticks require acaricide applications at higher concentrations and increased frequency. Acaricide resistance is a consequence of frequent selection of resistant individuals in tick populations in fields, after repetitive use of a single acaricide chemical class for long periods (CONWAY; COMINS, 1979).

Unfortunately, there is no official policy for cattle tick control in Brazil. Moreover, acaricides are frequently used irrationally, with few if any technical criteria for choosing the most appropriate product. Cattle tick control depends on acaricide efficiency in order to overcome the existing resistance to different classes of acaricides and on delaying the development of resistance towards new chemical classes, with adoption of rational acaricide application methods.

One of the most recommended strategies for avoiding resistance is to alternate the chemical acaricide groups used for controlling cattle tick populations (SUTHERST; COMINS, 1979). This strategy needs to be based on studying the acaricide resistance profile of the tick population of the location in question (i.e. the region or farm).

In Brazil, the most used synthetic pyrethroid insecticides are cypermethrin and deltamethrin. Over recent years, use of cypermethrin in association with organophosphates (OP) has increased with the aim of achieving better control.

Synthetic pyrethroid resistance was studied by Nolan et al. (1989) and Nolan (1990) in Australia. These authors characterized the resistance in three tick strains: Marmor (resistant to deltamethrin and cypermethrin, in the absence of flumethrin), Lamington (resistant to deltamethrin and cypermethrin, but susceptible to flumethrin) and Parkhurst (resistant to flumethrin, deltamethrin and cypermethrin).

In Brazil, synthetic pyrethroid resistance has been reported since 1980 in the states of Rio de Janeiro (LEITE, 1988), Rio Grande do Sul (FARIAS et al., 2008), Goiás (FERNANDES, 2001), Paraná (SOUZA et al., 2003), Bahia (CAMPOS Jr.; OLIVEIRA, 2005), Minas Gerais (FURLONG et al., 2007), São Paulo (MENDES et al., 2007) and Mato Grosso do Sul (GOMES et al., 2011).

Synthetic pyrethroids exert their acaricide effect in neuronal voltage-gated sodium channels, and mutations in these protein coding sequences can determine structural changes leading to reduced synthetic pyrethroid interaction, thus limiting their acaricide effect (SODERLUND; KNIPPLE, 2003).

Insensitivity of the active site, as determined by a point mutation (Phe replaced by Ile; T2134A) in the conserved IIS6 segment of the sodium channel of the San Felipe Mexican strain of *R. (B.) microplus* (which is highly resistant to DDT and permethrin), was identified by He et al. (1999). This mutation has also been found in many studies developed in Mexico (ROSARIO-CRUZ et al., 2005; 2009). In Brazil, however, this mutation was not found in resistant ticks from the states of Mato Grosso do Sul (ANDREOTTI et al., 2011), Minas Gerais (DOMINGUES et al., 2012), São Paulo, Espírito Santo, Paraná and Rio Grande do Sul (LOVIS et al., 2012). Jonsson et al. (2010) reported the mutation G214T in the domain II S4-S5, which is a glycine-to-valine change associated with resistance to the pyrethroid flumethrin.

The objectives of the present work were to diagnose synthetic pyrethroid resistance using standardized bioassays and to investigate occurrences of the T2134A mutation in field populations of *R. (B.) microplus* in the states of Rio Grande do Sul and Mato Grosso do Sul.

Materials and Methods

Ticks

Susceptible strain

The Mozo strain provided by the Department of Parasitology, "Miguel C. Rubino" Veterinary Laboratories Division, (DiLaVe), Ministry of Livestock, Agriculture and Fisheries (MGAP), Uruguay, was used as a reference susceptible strain in the diagnostic bioassays.

This strain was maintained in heifers at the Biological Institute of São Paulo. The animal handling was conducted in accordance with the certification granted by the Ethics Committee for Animal Experimentation of the Biological Institute (protocol number 24/06 CETEA-IB).

R. (B.) microplus field populations

Tick samples were collected in the years 2010 and 2011, from farms located in the states of Mato Grosso do Sul (n = 4) and Rio Grande do Sul (n = 6). Table 1 lists the tick populations analyzed, their respective origins and the previous acaricide treatments.

Tick preparation

The experiments were carried out following the FAO guidelines (2004) for the larval packet test. On each farm, engorged females (at least 4 mm each) were collected from at least 10 animals and taken to the laboratory at the Biological Institute of São Paulo. After being washed in water and dried on paper towels, these females were placed in a petri dish for egg laying. The containers were given identification and kept in an incubator at $27 \pm 1^\circ\text{C}$ and relative humidity of $80 \pm 5\%$ for 14 days. The eggs were

Table 1. Assignment of *R. (B.) microplus* populations analyzed and corresponding previous use of acaricides.

State	Strains (symbol)	Production purpose	Acaricides used (active components)
Mato Grosso do Sul	Canga (CG)	milk	deltamethrin; cypermethrin; fipronil and ivermectin
	Bonfim (BF)	milk	chlorpyrifos + cypermethrin in association
	Galheiro (GH)	meat	cypermethrin; cypermethrin + ethion in association
	Antônio (AT)	meat	cypermethrin; cypermethrin + ethion in association; amitraz
Rio Grande do Sul	Checo (CE)	—	—
	Pântano (PA)	meat	amitraz; fipronil; organophosphate; pyrethroid
	Gabriel (GB)	meat	macrocyclic lactones; amitraz; cypermethrin; chlorpyrifos
	Lousada (LS)	meat	fipronil; amitraz; cypermethrin; chlorpyrifos
	Cavalcanti (CT)	meat	fluazuron; ivermectin; cypermethrin; chlorpyrifos
	Cachoeira (CR)	meat	—

incubated in sealed glass vials, closed with wet cotton. The larvae used in the bioassays were between 14 and 21 days old.

Larval Packet Test (LPT)

The LPT was performed as prescribed by FAO (2004) with some minor modifications. The synthetic pyrethroids were used at their technical grades: cypermethrin (93.59% purity; Allvet), deltamethrin (99.2% purity; Coopers Animal Health) and flumethrin (97% purity; Sigma-Aldrich). The acaricides were dissolved in a vehicle composed of two parts of trichloroethylene and one part of olive oil. For each active ingredient, a dilution series was set up in order to obtain a concentration gradient resulting in 0 to 100% larval mortality.

The concentrations used were: flumethrin – 0.0079922%; 0.0133204%; 0.02202008%; 0.0275251%; 0.0344064%; 0.043008%; 0.05376%; 0.0672%; 0.084% and 0.12%; cypermethrin – 0.1269034%; 0.2115072%; 0.58752%; 0.9792%; 1.632% and 4%; and deltamethrin – 0.085%; 0.107%; 0.134%; 0.167%; 0.209%; 0.262%; 0.327%; 0.409%; 0.512%; 0.64% and 0.8%. For each dilution series, a negative control was used (composed only of the vehicle). For each concentration, three repetitions were made. A volume of 670 µL of each solution was applied to Whatman No. 1 (7.5 × 8.5 cm) filter paper.

After saturation, these paper filters were left for 24 hours at room temperature for trichloroethylene evaporation. Approximately 100 larvae were transferred to each packet using a paintbrush, and the packet was closed with paper clips and kept in an incubator (27 ± 1 °C and 80% ± 5% relative humidity). After 24 hours, the packets were opened, and both the live and the dead larvae were counted, with the help of a vacuum pump and a manual counter. The ability of the larvae to walk on the surface of the filter paper was used as the criterion for determining whether larvae were dead or alive.

Data analysis

The percentage mortality for each concentration was calculated as the number of dead larvae relative to the total number of larvae.

The data obtained from the tests were submitted to statistical analysis by means of the Polo Plus software (LeOra Software, 2004).

These analyses provided the LC₅₀ and LC₉₉ for each population (lethal concentrations for 50% and 99% of the population, respectively) with 95% confidence intervals, along with the slopes of the regression lines. The resistance factor (RF) of each population studied was calculated by dividing the LC₅₀ of the field population by the mean LC₅₀ of the assays performed with the susceptible reference strain (Mozo).

DNA extraction

Thirty larvae from each population were subjected to DNA extraction as described by Guerrero et al. (2001). Briefly, larvae were placed individually in 1.5 mL microcentrifuge tubes containing 20 mM Tris-HCl (pH 8.4) and 50 mM KCl 50 (Invitrogen). The larvae were crushed using a round-pointed pestle, and the resulting suspension was heated to 100 °C for 3 minutes, and used directly in the PCRs, or was frozen at –20 °C until use.

Polymerase chain reaction

To detect the mutation T2134A, the protocol developed by Guerrero et al. (2001) was used. In each reaction, reagents were distributed at the following concentrations: PCR buffer of 20 mM Tris-HCl (pH 8.4) and 50 mM KCl (Invitrogen), dNTPs (0.2 mM each), MgCl₂ (2 mM), Dream Taq DNA Polymerase® (1 µL) (Fermentas), along with 2.5 µL of the larva suspension. To amplify susceptible alleles, the following primers were used: FG221 (5'–TTATCTTGGCTCCTTCT–3') and FG227 (5'–TTGTTTCATTGAAATTGTCGA–3'). To amplify resistant alleles, the following primers were used: FG222 (5'–TTATCTTCGGCTCCTTCA–3') and FG227 (5'–TTGTTTCATTGAAATTGTCGA–3'). Each of these was used at a concentration of 0.4 µM.

The conditions programmed in the TC-02024 Multigene™ mini thermal cycler (Labnet International®) that was used to amplify the gene fragments were as follows: 94 °C for 5 min, 40 cycles of 94 °C for 45 sec, 60 °C for 30 sec and 72 °C for 40 sec,

with a final step of 72 °C for 7 min. The amplified fragments were subjected to electrophoresis on 3% agarose gel stained with Sybr Safe Gel Stain™ (Life Technologies®), and were analyzed on a UV transilluminator.

Susceptible and resistant-allele controls were included through addition of a sample containing larval genomic DNA from the Mexican strain San Felipe (heterozygous for the T2134A mutation).

Results

All of the tick samples analyzed showed resistance to cypermethrin (Table 2). The RFs ranged from 5.6 to 80.3 in Rio Grande do Sul and from 8.8 to 28.3 in Mato Grosso do Sul.

As observed for cypermethrin, all of the tick populations analyzed were resistant to deltamethrin (Table 3), with RFs from 2.4 to 83.1 in Rio Grande do Sul and from 18.7 to 47.9 in Mato Grosso do Sul. The slope values were all below 3.2.

Six of the eight populations on which flumethrin had been used (Table 4) showed resistance, with RFs from 3.8 to 8.2. Two populations were susceptible to flumethrin: one from the state of Mato Grosso do Sul (RF = 0.4) and the other from Rio Grande

do Sul (RF = 1.4). In the Mato Grosso do Sul samples, the lowest RF found was 5.6 and the highest, 8.2. In the state of Rio Grande do Sul, the RF for flumethrin ranged from 6.4 to 7.2.

The PCR results showed that none of the 10 populations analyzed possessed the mutant allele T2134A.

Discussion

Today in Brazil, commercial acaricides containing flumethrin as the active compound are no longer available, in spite of its past widespread use in the 1980s and 1990s in association with the organophosphate coumaphos. This may account for the lower RFs calculated for flumethrin (3.8 to 8.2), in comparison with those calculated for cypermethrin (5.6 to 80.3) and deltamethrin (2.4 to 83.1) (Tables 2, 3 and 4).

Since flumethrin-based acaricides had not been used for over a decade, one important aspect of the present study was to detect lateral resistance among the synthetic pyrethroids analyzed. Seven of the eight populations that had been exposed to flumethrin showed resistance towards this compound. In Australia, Nolan et al. (1989) described three strains with different patterns of resistance

Table 2. *R. (B.) microplus* susceptibility to cypermethrin (larval packet test) in samples from the states of Mato Grosso do Sul and Rio Grande do Sul.

State	Strain	n	Slope (SD)	LC ₅₀ (95% CI)	LC ₉₉ (95% CI)	RF ₅₀
-	Mozo	13353	4.132 (0.068)	0.0147 (0.014-0.0155)	0.1374 (0.1153-0.1691)	
MS	CG	1091	1.86 (0.107)	0.346 (0.308-0.386)	6.159 (4.648-8.723)	23.5
MS	BF	1096	2.248 (0.138)	0.2 (0.172-0.228)	2.171 (1.688-3.001)	13.6
MS	GH	995	1.764 (0.138)	0.13 (0.105-0.154)	2.712 (1.971-4.149)	8.8
MS	AT	971	2.024 (0.114)	0.417 (0.33-0.519)	5.876 (3.534-12.874)	28.3
RS	CE	2185	3.229 (0.159)	1.18 (1.098-1.266)	6.197 (5.308-7.455)	80.3
RS	PA	1509	3.155 (0.134)	0.467 (0.433-0.504)	2.552 (2.173-3.092)	31.8
RS	GB	2555	2.446 (0.078)	0.683 (0.615-0.755)	6.101 (4.855-8.078)	46.5
RS	LS	491	2.088 (0.288)	0.09 (0.057-0.12)	1.17 (0.807-2.157)	6.1
RS	CT	1946	2.648 (0.109)	0.48 (0.407-0.557)	3.627 (2.719-5.367)	32.6
RS	CR	1899	1.891 (0.156)	0.082 (0.049-0.111)	1.395 (0.9341-2.801)	5.6

MS: Mato Grosso do Sul; RS: Rio Grande do Sul; n: number of larvae; SD: standard deviation; LC: lethal concentration; 95% CI: 95% confidence interval; RF: resistance factor.

Table 3. *R. (B.) microplus* susceptibility to deltamethrin (larval packet test) in samples from the states of Mato Grosso do Sul and Rio Grande do Sul.

State	Population	n	Slope (SD)	LC ₅₀ (95% CI)	LC ₉₉ (95% CI)	RF ₅₀
	Mozo	2349	2.61 (0.095)	0.00373 (0.0028-0.005)	0.02902 (0.015-0.095)	
MS	CG	2141	1.531 (0.115)	0.07 (0.058-0.084)	2.365 (1.708-3.65)	18.7
MS	BF	1828	2.838 (0.191)	0.084 (0.075-0.093)	0.555 (0.475-0.679)	22.5
MS	GH	1959	2.93 (0.163)	0.104 (0.096-0.112)	0.65 (0.563-0.774)	27.8
MS	AT	1965	3.114 (0.136)	0.179 (0.169-0.188)	0.998 (0.879-1.158)	47.9
RS	CE	1660	2.585 (0.156)	0.31 (0.265-0.366)	2.458 (1.65-4.407)	83.1
RS	PA	2567	3.114 (0.111)	0.2 (0.191-0.208)	1.115 (0.996-1.27)	53.6
RS	GB	2872	3.001 (0.109)	0.197 (0.189-0.205)	1.172 (1.04-1.343)	52.8
RS	LS	566	2.744 (0.331)	0.07 (0.053-0.084)	0.491 (0.382-0.725)	18.7
RS	CT	2103	3.075 (0.142)	0.127 (0.112-0.14)	0.723 (0.595-0.937)	34
RS	CR	1591	1.588 (0.115)	0.009 (0.004-0.014)	0.261 (0.115-2.007)	2.4

MS: Mato Grosso do Sul; RS: Rio Grande do Sul; n: number of larvae; SD: standard deviation; LC: lethal concentration; 95% CI: 95% confidence interval; RF: resistance factor.

Table 4. *R. (B.) microplus* susceptibility to flumethrin (larval packet test) in samples from the states of Mato Grosso do Sul and Rio Grande do Sul.

State	Population	n	Slope (SD)	LC ₅₀ (95% CI)	LC ₉₉ (95% CI)	RF ₅₀
	Mozo	2099	1.073 (0.095)	0.005 (0.002-0.007)	0.674 (0.34-2.129)	-
MS	CG	1881	2.311 (0.112)	0.028 (0.025-0.03)	0.281 (0.212-0.407)	5.6
MS	BF	2222	3.121 (0.129)	0.019 (0.018-0.021)	0.107 (0.089-0.135)	3.8
MS	GH	1786	1.098 (0.133)	0.002 (0-0.005)	0.259 (0.118-2.017)	0.4
MS	AT	1800	2.593 (0.119)	0.041 (0.037-0.045)	0.32 (0.239-0.474)	8.2
RS	PA	1465	2.697 (0.147)	0.034 (0.031-0.038)	0.251 (0.183-0.393)	6.8
RS	GB	1408	2.696 (0.148)	0.036 (0.032-0.04)	0.26 (0.186-0.424)	7.2
RS	LS	548	1.953 (0.228)	0.007 (0.005-0.01)	0.114 (0.082-0.187)	1.4
RS	CT	2161	2.975 (0.15)	0.032 (0.028-0.036)	0.192 (0.0141-0.307)	6.4

MS: Mato Grosso do Sul; RS: Rio Grande do Sul; n: number of larvae; SD: standard deviation; LC lethal concentration; 95% CI: 95% confidence interval; RF: resistance factor.

to synthetic pyrethroids: Parkhurst strain, resistant to flumethrin, deltamethrin and cypermethrin; Marmor strain, resistant to deltamethrin and cypermethrin but susceptible to flumethrin; and Lamington strain, resistant to flumethrin but susceptible to cypermethrin. Therefore, the resistance pattern found in the populations assessed in the present study correspond to the Parkhurst strain. Regarding flumethrin, the GH population from Mato Grosso do Sul presented LC₅₀ (0.002%) that was very close to the LC₅₀ of the Yeerongpilly strain (0.0026%)

All of the populations from the state of Mato Grosso do Sul had previously been exposed to cypermethrin. Only the CG population had previously been exposed to both deltamethrin and cypermethrin. This information may account for the tick resistance to synthetic pyrethroids in all of the populations analyzed, which confirms the previous results of Gomes et al. (2011) in this state, who found cypermethrin efficacy towards *R. (B.) microplus* adults of only 19.94%.

The data from the present study emphasize the high frequency of resistance observed in the state of Rio Grande do Sul. According to Jonsson et al. (2007), the larval packet test (LPT) provides better precision of information about the resistance.

There is a consensus that resistance among cattle ticks to synthetic pyrethroids is currently widespread in Brazil, and therefore that synthetic pyrethroid-based formulations must be used with caution and only in absence of resistance.

The PCR results obtained were in agreement with those found by Andreotti et al. (2011), Domingues et al. (2012) and Lovis et al. (2012), which demonstrated absence of the point mutation (T2134A) conferring SP resistance. Morgan et al. (2009) found another non-synonymous point mutation (L64I) in *R. (B.) microplus* populations resistant to SP in Australia. Thus, it is plausible that another mutation is also associated with resistance to synthetic pyrethroids in Brazil.

Use of molecular diagnostic tools might provide valuable information for cattle tick management and control in the field, since these make it possible to monitor the frequencies of resistant alleles in order to forecast accumulations of resistant or susceptible allele over defined periods of time.

Prior susceptibility evaluation on the tick population is essential, in order to adjust the acaricide use to the specific characteristics of each farm. In this manner, better acaricide efficiency can be

achieved, with the aim of reducing selection pressure to be as weak as possible. In order to follow all of these criteria, field education is another essential factor to be taken into consideration.

Conclusions

This was the first detection of flumethrin resistance in Brazil. The T2134A mutation was not found in the populations studied.

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