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Resistance profile of *Rhipicephalus (Boophilus) microplus* to diazinon and cypermethrin and first report of sodium channel mutation - domain III S6 - T2134A, in field samples from the state of São Paulo, Brazil

[*Perfil de resistência do* Rhipicephalus (Boophilus) microplus *ao diazinon e à cipermetrina e primeiro relato de mutação no canal de sódio - domain III S6 - T2134A em amostras de campo, no estado de São Paulo - Brasil*]

M.L. *Santos*[1], L.C. *Fiorini*[1], F.C. *Duarte*[1], K.A. *Anjos*[1], A.F.C. *Nassar*[1], L.G. *Brito*[2], J.R. *Pereira*[3], M.C. *Mendes*[1*]

[1]Instituto Biológico, Laboratório de Parasitologia Animal, São Paulo, Brasil
[2]Embrapa Amazônia Oriental, Belém, PA, Brasil
[3] Polo Regional Vale do Paraíba. APTA, Pindamonhangaba, SP, Brasil

ABSTRACT

Rhipicephalus (Boophilus) microplus is one of the parasites that has an impact on livestock farming in Brazil. Bioassays using the larval packet test (LPT) impregnated with cypermethrin and diazinon at different concentrations were performed to characterize phenotypic resistance (resistance level, RL), molecular resistance (mutation in domain III - S6 T2134A) and enzymatic metabolism of diazinon and cypermethrin in some populations of *R. microplus* that were collected in different regions of the state of São Paulo. Among the 40 farms analyzed, 18 of them presented resistance factors for cypermethrin, with RL I (53%) and RL II (47%). Mutation of domain III T2134A was found for the first time in Brazil, on seven farms. Heterozygous larvae were found on six of them and resistant homozygous larvae on four. No differences ($p>0.05$) in enzymatic activity of α-esterase and β-esterase were found in tests with live and dead larvae at a cypermethrin concentration of 409.6μg/cm^2. Ninety percent of the farms showed resistance to the active agent diazinon: RL I in 6%, RL II in 30% and RL III in 64%. There were significant differences ($p<0.05$) in enzymatic activity at the highest concentration (3.2μg/cm^2).

Keywords: ticks, pyrethroids, organophosphate

RESUMO

Rhipicephalus (Boophilus) microplus (Canestrini) é um dos parasitas que mais impactam a pecuária de países tropicais e subtropicais, incluindo o Brasil, com perdas em torno de US$ 3,24 bilhões por ano. Ocorrências de populações resistentes a diferentes classes de acaricidas e suas associações têm sido amplamente diagnosticadas em todo o Brasil. Para isso, bioensaios utilizando o teste de pacote larval (LPT) impregnado com cipermetrina e diazinon em diferentes concentrações foram realizados para caracterizar a resistência fenotípica (nível de resistência, RL), a resistência molecular (mutação no domínio III - S6 T2134A) e o metabolismo enzimático do diazinon e da cipermetrina em algumas populações de R. (Boophilus) microplus coletadas em diferentes regiões do estado de São Paulo. Dentre as 40 propriedades analisadas, 18 delas apresentaram fatores de resistência à cipermetrina, sendo RL I (53%) e RL II (47%). A mutação do domínio III T2134A foi encontrada pela primeira vez no Brasil, em sete fazendas. Larvas heterozigotas foram constatadas em seis delas, e larvas homozigotas resistentes em quatro. Nenhuma diferença (P>0,05) na atividade enzimática de α-esterase e β-esterase foi observada em testes com larvas vivas e mortas em uma concentração de cipermetrina de 409,6μg/cm^2. Noventa por cento das propriedades apresentaram resistência ao agente ativo diazinon: RL I em 6%, RL II em 30% e RL III em 64%. Houve diferenças significativas (P<0,05) na atividade enzimática na concentração mais elevada (3,2μg/cm^2) entre os grupos de larvas vivas e mortas.

Palavras-chave: carrapatos, piretroides, organofosforado

INTRODUCTION

The financial losses caused by the tick *R. microplus* are estimated around US$ 3.24 billion per year. These are generated by loss of animals due to cattle tick fever, dermatitis caused by the presence of the ectoparasite, blood loss and, consequently, weight loss and reduction of milk production. In addition, there is expenditure due

*Corresponding author: marcia.mendes@sp.gov.br
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to control measures that are implemented (Grisi *et al*., 2014).

Chemical treatment is the main means of controlling *R. microplus* in cattle. The main chemical groups used are organophosphates, pyrethroids, formamidines, macrocyclic lactones, phenylpyrazoles and fluazuron. Resistance of ticks to these groups is widespread in Brazil. In the state of São Paulo, all these acaricide classes are used (Lovis, 2013; Higa *et al*., 2016). Fourteen years ago, the products most used by farmers for treating cattle consisted of associations of pyrethroids and organophosphates. This was especially so in the Paraíba valley region, which is considered to be one of the largest milk-producing regions of the state of São Paulo (Mendes *et al*., 2007).

Metabolic resistance is conferred by a metabolic process in which insects are able to detoxify or break down the toxin faster than susceptible insects. It is known that some enzymatic systems may be involved in biotransformation and excretion of insecticides in resistant strains and may have higher levels of expression over a broad spectrum of activity relating to detoxification (Guidelines…, 2004).

Four families of enzymes are associated with insecticide metabolism: carboxylesterases (CCEs), glutathione S-transferases (GSTs), uridine 5'-diphospho-glucuronosyltransferases (UDP-glucuronosyltransferase, UGTs) and cytochrome P450s (CYP450). These have been implicated in conferring resistance to one or more classes of insecticides (Rosario-Cruz *et al.*, 2009; Cossío-Bayogar *et al*., 2018; Feyereisen *et al*., 2015).

Brito *et al*. (2017) used a polymerase chain reaction (qPCR) to quantify the enzymes acetylcholinesterase and esterase in diazinon-resistant *R. microplus* strains that were obtained from Pirajuí and Presidente Médice, RO, Brazil. Chigure *et al*. (2017) studied the correlation between resistance and functional enzymes in *R. microplus* populations in India. Several research groups have focused on characterization of acaricide resistance through bioassays and development of new diagnostic methodologies, such as molecular tests to quickly detect resistance with the objective of directing correct management of acaricides.

As already known, non-metabolic resistance occurs at acaricide target sites through nucleotide substitutions in the gene, which lead to insensitivity of the target site to acaricide. According to Narahashi (2002), the target site for pyrethroids is the sodium channel that is present in the cells of the central and peripheral nervous system. This targeting results in prolonged opening of individual channels, which causes paralysis and death of these arthropods.

Kumar *et al*. (2020) presented research data from different locations around the world and reported that at least ten single-point mutations (single nucleotide polymorphisms, SNPs) that replaced the sodium channel gene had been identified. These authors considered that five non-synonymous mutations out of these ten (T170C, C190A, G215 T, T2134A and T2134C) were clearly associated with resistance to pyrethroids. On the other hand, five other SNPs (non-synonymous mutations C148 T, G184C and C190 G and synonyms C189A and C2130 T) did not seem to be related to resistance. In Brazil, the C190A mutation in domain II S4-5 (resulting from substitution of amino acids from leucine to isoleucine) was the only mutation reported in tick populations that were resistant to pyrethroids.

The present study was carried out to characterize phenotypic and molecular resistance (mutation in domain III - S6 T2134A) and enzymatic metabolism of diazinon and cypermethrin in populations of ticks collected in different regions of the state of São Paulo.

MATERIALS AND METHODS

This study was conducted on the premises of the Animal Parasitology Laboratory and the General Bacteriology Laboratory of the Animal Health Research Center of the Biological Institute, São Paulo, SP (latitude: 23º 32' 51" S; longitude: 46º 38' 10" W; and altitude: 760m) in the years 2019 and 2020.

Female ticks were collected from animals on 40 farms distributed in 23 municipalities in different regions of the state of São Paulo: Águas de São Pedro (22º 35' 58" S; 47º 52' 34" W); Andradina (20º 53' 46" S; 51º 22' 46" W); Atibaia (23º 07' 01" S; 46º 33' 01" W); Batatais (20º 53' 28" S; 47º 35' 06" W); Bauru (22º 18'53" S; 49º 03' 38" W); Bragança Paulista (22º 57' 07" S; 46º 32' 31"

W); Brotas (22º 17' 03" S; 48º 07' 36" W); Castilho (20º 52' 20" S; 51º 29' 15" W); Cunha (23° 05' 03" S; 44° 57' 40" W); Guaratinguetá (22º 48' 59" S; 45º 11' 33" W); Itapetininga (23º 35' 30" S; 48º 03' 11" W); Joanópolis (22º 55' 49" S; 46º 16' 32" W); Lorena (22º 43' 51" S; 45º 07' 29" W); Monte Mor (22º 56' 48" S; 47º 18' 57" W); Murutinga do Sul (20º 59' 36" S; 51º 16' 39" W); Nazaré Paulista (23° 10' 42" S; 46° 23' 51" W); Piedade (23º 42' 43" S; 47º 25' 40" W); Piracaia (23º 03' 14" S; 46º 21' 29" W); Potim (22º 50' 34" S; 45º 15' 05" W); São Miguel Arcanjo (23º 52' 42" S; 47º 59' 50" W); Sarapuí (20º 53' 46" S; 51º 22' 46" W); Sorocaba (23° 30' 06.01" S; 47° 27' 29.02" W); and Socorro (22º 35' 29" S; 46º 31' 44" W).

In the laboratory, the female ticks were kept in an incubation chamber at 28°C and 80% humidity for oviposition to take place. After this, the eggs were packed into tubes (5.3cm high by 2.4cm in diameter) that were closed with dampened cotton fiber, to await hatching of the larvae. These larvae were later used to perform resistance tests using the technique Stone and Haydock (1962).

Whatman™ no. 1 filter papers (7.5 x 8.5cm) were impregnated in the laboratory of Embrapa Amazônia Oriental, in Belém, PA, Brazil (1° 27' 18" S; 48° 30' 09" W), with the active ingredients diazinon (concentrations: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, 2.4 and 3.2µg/cm^2) and cypermethrin (concentrations: 1.6, 6.4, 25.6, 102.4 and 409.6µg/cm^2), both at analytical standard ≥ 90.0% purity (Merck Sigma Aldrich® Co., St. Louis, MO, USA). A control was formed by impregnating the filter paper only with the solvent acetone.

Approximately one hundred larvae of *R. microplus* were transferred to each packet using a brush. The packets were then sealed with clips and incubated in an incubation chamber at 28°C and 80% relative humidity. These tests were performed in triplicate. After 24 hours, the dead and living individuals were counted. Larvae that were paralyzed or only moving their appendages, without the ability to walk, were considered dead.

The mortality data from the larval packet test were analyzed using the "probits" module of the POLO-PC software (Leora Software, 1987). The resistance level (RL) classification was calculated as described by Mendes *et al*. (2007), which distributes the resistance levels according to the chart below:

Presence or absence of the T2134A mutation of domain III was ascertained using individual larvae of samples P10, P11, P14, P15, P16, P19, P20, P27, P36 and P37 (sample size "n" given on Table 2), in accordance with the protocol of Guerrero *et al*. (2001). Genomic DNA was extracted using the Quick-DNA™ Miniprep Plus extraction kit (D4069; Zymo Research®), following the manufacturer's guidelines.

For amplification of mutant kdr alleles, the primers for resistant alleles equivalent to 221R, was used: 5' - TTATCTTCGGCTCCTTCA - 3'. For amplification of wild-type kdr alleles, the primer for susceptible alleles, equivalent to 221S, was used: 5' – TTATCTTCGGCTCCTTCT – 3'. The nonspecific reverse primer, equivalent to 227I, was also used: 5' – TTGTTCATTGATGATGTCGA – 3'. In addition, negative and positive controls were added: the negative control was prepared replacing DNA template by ultrapure water, and the positive PCR control was performed using a chemically synthesized DNA stretch of 68 base pairs corresponding to the resistant alelle: 5' – TTATCTTCGGCTCCTTCATCACCTTGAATC TATTCATCGGTGTTATTATCGACAATTTC AATGAACAA – 3'

To perform the polymerase chain reaction (PCR) a volume of 22.5µL was used, containing 2µL of the target DNA solution, 1µL 10 pM/µL of the primer for the resistant kdr allele (221S) and 1µL 10pM/µL of the nonspecific reverse primer (227I); or 1µL of the primer for the susceptible kdr allele (221R) and 1µL 10 pM/µL of the nonspecific reverse primer (227I). In addition, the volume contained 12.5µL of the TaqDNA polymerase 2x Master Mix RED™ amplification kit (Ampliqon®, Odense, Denmark), consisting of Tris-HCl (pH 8.5), $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.2% Tween™ 20, 0.4mM deoxynucleotide triphosphates (dNTP) and 8.2µl of ultrapure water (Invitrogen®, Carlsbad, CA, USA).

The reactions were performed at 96°C for 2min, followed by 42 PCR cycles (94°C for 1min, 58°C for 1min and 72°C for 1min) and then a final extension at 72°C for 7min. The amplicons

obtained from the PCR were visualized on 3% agarose gel stained with UniSafe Dye™ Nucleic Acid staining solution (20,000x) (Uniscience® Corporation, Miami Lakes, FL 33015, USA).

The enzymatic activity was tested with five surviving larvae and five dead larvae that were removed from the filter paper impregnated with the highest concentration of each product. In relation to diazinon, this test was performed on fifteen RL III farms, while for cypermethrin it was performed on three RL I and six RL II farms.

The enzymatic assays were carried out in accordance with the micro burette method. To determine the total protein concentration, two reagents were used: copper sulfate 6mmol/L (reagent 1) and NaOH 1.15mol/L (reagent 2). Each sample was homogenized in two replicates, using 500µL of each reagent, 995µL of distilled water and 5µL of the sample. The standard used was bovine serum albumin at a concentration of 50g/L. Absorbance measurements at 570nm were made in a Femto 600 plus spectrophotometer and the results were expressed in g/L.

The enzymatic activity of the esterase was determined through addition of α and β-naphthyl. In each well, 20µL of the homogenate supernatant (for both α and β-esterase) was added to 250µL of α/β-25 naphthyl acetate solution dissolved in 24.75mL of 20 mM sodium phosphate buffer (pH 7.2). The reaction was incubated at room temperature for 30 minutes. Then, 50µL of Fast Blue B solution (0.045g of Fast Blue B) was added to 4.5mL of distilled water that had been added to a solution of 15mL of 5% SDS. The reaction was incubated at room temperature for another 5 minutes and then 200µL of α/β-naphthyl acetate/sodium phosphate and another 50µL of Fast Blue were added. Absorbance readings were made at 570nm.

Analysis of variance was performed to determine any differences in enzymatic activity between live and dead larvae for each active agent. The Tukey test was used to compare the means, taking the significance level to be 5%.

RESULTS

The active agents used on each farm and the resistance data on the products cypermethrin and diazinon are presented in Tables 1 and 3. Use of pyrethroids and organophosphates was cited by 52.5% of the farm owners, and 20% reported using unassociated pyrethroids. Other chemical groups were mentioned with the following frequencies: 15% formamidine, 12.5% fluazuron, 10% macrocyclic lactones, 7.5% homeopathy, 5% unassociated organophosphates and 2.5% phenylpyrazoles; and 17.5% did not mention any product.

Table 1 presents test results using cypermethrin. The resistance factor was evaluated using LC_{50} from farm P2 (LC_{50}: 1.08), used as susceptible. Among the 40 farms analyzed, eighteen of them presented resistance factors ranging from 1.58 to 28.8, with RL I (10 farms) and RL II (8 farms). Out of these eighteen resistant samples, only five of them did not report use of cypermethrin (alone or in association with another agent) for treating animals against *R. microplus.* These resistance levels indicate if the acaricide resistance is still building up or if it is already established.

Mutation of domain III T2134A of the ten samples of *R. microplus* analyzed (Table 2) was identified on seven farms (60% heterozygous and 40% homozygous). In the P10 sample, with resistance ratio of 10.3, 87.5% of the larvae presented heterozygous resistance. The P19 sample, with resistance ratio of 28.2, showed larvae with homozygous resistance for mutation III T2134A (Figure 3).

Table 1. Characterization of the phenotype of resistance and susceptibility to cypermethrin in a population of *Rhipicephalus (Boophilus) microplus* larvae in the state of São Paulo

Classification	Pyrethroids – Resistance Factor	Organophosphate – Resistance Factor
Sensitive	≤2.4	< 1.4
Resistant level I	2.5 – 5.4	1.5 – 4.4
Resistant level II	5.5 – 50	4.5 -50
Resistant level III	>50	> 50

N = number of individuals; LC_{50} (95% CI) = concentration that kills 50%, with confidence interval; RF = resistance factor; RL = resistance level

Table 2. Frequency of mutation of domain III T2134A *R. microplus* samples from some regions of São Paulo, Brazil

	Municipalities	Active agents	N	LC_{50} (95% CI)	SLOPE±SE	RF	RL
P1	São Pedro	Not informed	1388	-	0.35±0.181	-	-
P2	São Pedro	Fluazuron + abamectin	2858	1.08(0.252-1.626)	2.8±0.272		-
P3	São Pedro	No treatment used	2818	1.71(1.344-2.075)	2.89±0.211	1.58	I
P4	São Pedro	Not informed	1652	-	0.33±0.277	-	-
P5	Monte Mor	Cypermethrin, amitraz, chlorpyrifos, fenthion, dichlorvos	1595	-	13.88±754198	-	-
P6	Bragança Paulista	Cypermethrin + chlorpyriphos + citronella; diflubenzuron	2271	-	14.89±7193.20	-	-
P7	São Pedro	Not informed	2046	-	16.42±23281.0	-	-
P8	Nazaré Paulista	Cypermethrin + chlorpyrifos + citronella; diflubenzuron; doramectin	2676	-	0.77±0.048	-	-
P9	Bragança Paulista	Not informed	2077	-	13.011±4359.7	-	-
P10	Atibaia	Cypermethrin + chlorpyrifos + citronella; amitraz	2795	11.2(7.151-16.71)	0.91±0.036	10.3	II
P11	Atibaia	Cypermethrin + chlorpyrifos + citronella; deltamethrin	2499	7.40(6.249-8.701)	2.37±0.092	6.85	II
P12	Nazaré Paulista	diflubenzuron; doramectin	2487	-	14.38±7281.21	-	-
P13	Socorro	Amitraz; cypermethrin + chlorpyrifos + citronella	2469	-	14.49±10564.4	-	-
P14	Guaratinguetá	Cypermethrin; fluazuron	2050	5.29(4.395-6.238)	3.23±0.188	4.89	I
P15	Guaratinguetá	Cypermethrin + chlorpyrifos + citronella; amitraz	1921	3.67(3.357-3.977)	3.28±0.202	3.39	I
P16	Guaratinguetá	Cypermethrin + chlorpyrifos + citronella; fluazuron; flumethrin	2168	5.01(4.482-5.601)	2.47±0.103	4.63	I
P17	Guaratinguetá	Cypermethrin + chlorpyrifos + citronella	807	-	1.62±0.134	-	-
P18	Guaratinguetá	Cypermethrin + chlorpyrifos + citronella; amitraz	796	-	0.67±0.059	-	-
P19	Guaratinguetá	Flumethrin; cypermethrin + chlorpyrifos + citronella; amitraz	2825	30.5(21.572-43.7)	1.03±0.034	28.8	II
P20	Guaratinguetá	Cypermethrin + chlorpyrifos + citronella; amitraz	2817	8.65(7.056-10.51)	3.22±0.126	8.0	II
P21	Guaratinguetá	Cypermethrin + chlorpyrifos + citronella; fipronil	2249	2.92(2.117-3.967)	4.35±0.209	2.7	I
P22	Andradina	Cypermethrin; chlorpyrifos	2275	-	14.64±73431.7	-	-
P23	Murutinga do Sul	Cypermethrin + chlorpyrifos; citronella	744	-	12.12±16308.9	-	-
P24	Castilho	Cypermethrin; chlorpyrifos	540	2.85(2.410-3.402)	2.89±0.326	2.63	I
P25	Murutinga do Sul	Cypermethrin + chlorpyrifos; Dichlorvos + cypermethrin	626	-	13.73±17033.0	-	-
P26	Murutinga do Sul	Cypermethrin + chlorpyrifos + citronella	1674	3.16(2.630-3.840)	4.36±0.233	2.92	I
P27	Murutinga do Sul	Cypermethrin + chlorpyrifos + citronella; deltamethrin; ivermectin	1844	6.42(4.835-8.592)	2.59±0.118	5.94	II
P28	Murutinga do Sul	Cypermethrin + chlorpyrifos + citronella;	874	-	14.39±20247.6	-	-
P29	Murutinga do Sul	Not informed	890	-	27.83±40312.4	-	-
P30	Guaratinguetá	Cypermethrin; fenthion; chlorpyrifos	2694	-	15.28±14105.6	-	-
P31	Guaratinguetá	Cypermethrin + chlorpyrifos; citronella	2432	12.2(8.229-18.28)	4.132±0.192	11.2	II
P32	Brotas	Not informed	625	-	0.00±9796.806	-	-
P33	Brotas	Not informed	760	-	1.310±0.088	-	-
P34	Guaratinguetá	Cypermethrin + chlorpyrifos + citronella; amitraz	2470	-	14.66±4608.79	-	-
P35	Itapetininga	Homeopathy	2433	3.05(2.509-3.656)	3.08±0.147	2.82	I
P36	Itapetininga	homeopathy	1163	15.9(6.220-36.23)	0.68±0.048	14.7	II
P37	Sarapuí	Homeopathy	1178	8.94(1.575-28.62)	1.22±0.060	8.27	II
P38	Piedade	Cypermethrin	1671	2.48(0.877-4.333)	2.09±0.125	2.29	I
P39	Ribeirão Preto	Not informed	1494	-	0.90±0.048	-	-
P40	Capivari	Not informed	1441	4.93(0.779-11.93)	1.26±0.067	4.56	I

RF: resistance factor; RS: Resistant heterozygous; RR: Resistant homozygote; SS: sensitive; n: number of larvae; % freq.: percentage frequency.

Graphs of the enzymatic activity of α-esterase and β-esterase, tested with live and dead larvae at a cypermethrin concentration of 409.6μg/cm^2, from samples P10, P11, P14, P15, P16, P19, P20, P27, P36 and P37, did not show any significant difference between the two groups ($p>0.05$) (Figures 1 and 2).

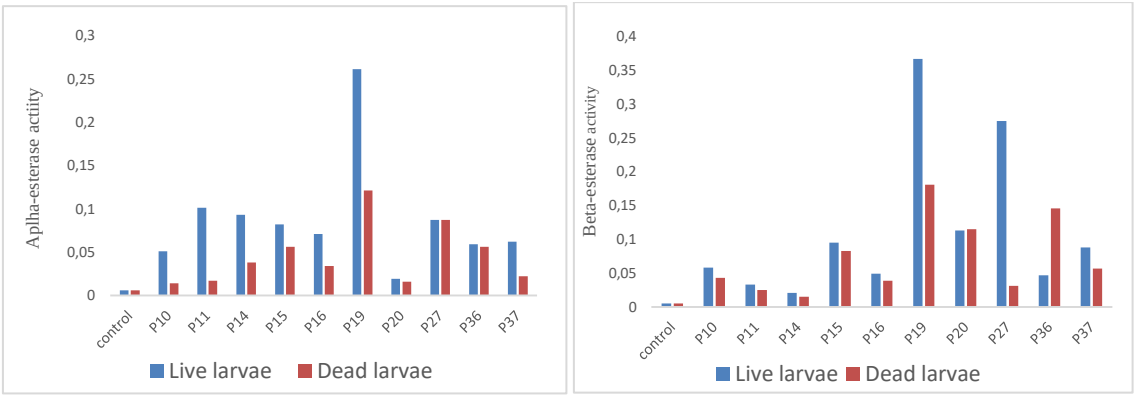


Figure 1. Evaluation of enzymatic activity of α-esterase and β-esterase in *R. microplus* larvae (populations from P5 to P40) exposed to cypermethrin. Control = larvae that were not exposed to cypermethrin.

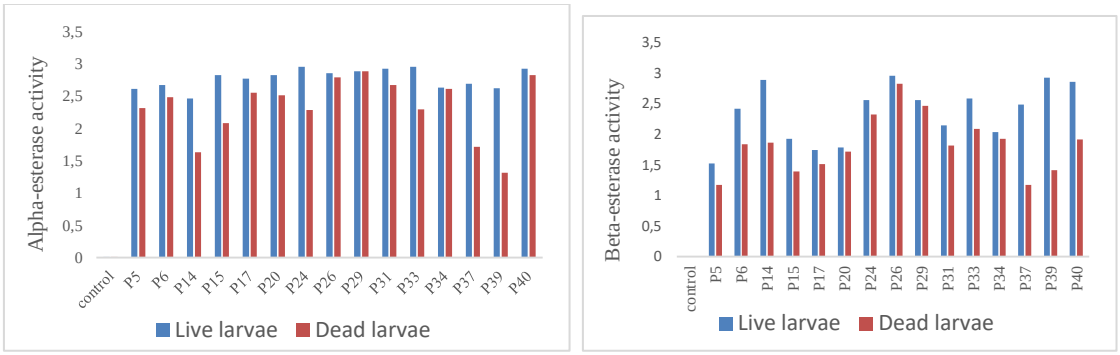


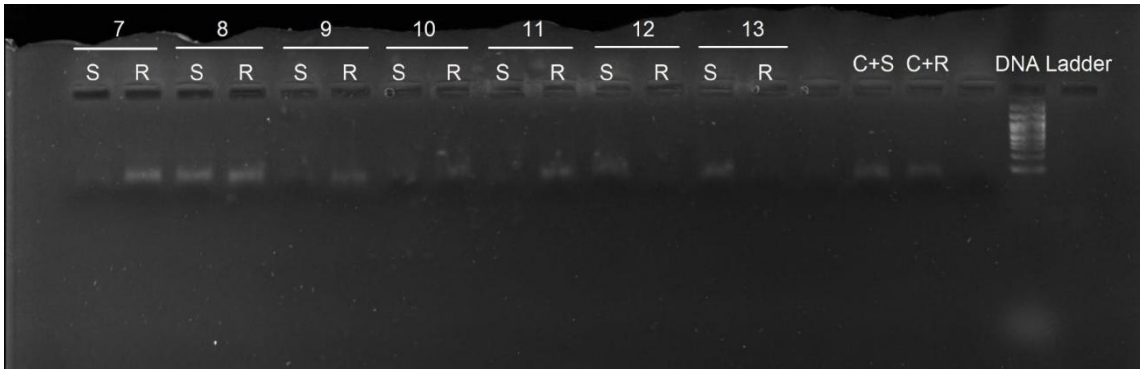
Figure 2. Evaluation of the enzymatic activity of α-esterase and β-esterase in *R. microplus* larvae (populations from P5 to P40) exposed to diazinon. Control = larvae that were not exposed to organophosphates.

Table 3 presents the results from *R. microplus* tests with the active agent diazinon. 90% of the farms presented resistance, taking the P3 sample as the susceptibility standard (LC_{50}: 0.03). The RL percentages found were 6% with RL I, 30% with RL II and 64% with RL III. It was observed that the farms with RL II and III presented percentages of organophosphate use of 46.6% and 69.5%, respectively, in association with another active agent, for treating cattle. The results regarding enzymatic P34, P37, P39 and P40) demonstrated significant differences ($p<0.05$) between the groups of live and dead larvae.

Table 3. Characterization of the phenotype of resistance and susceptibility to diazinon in a population of *Rhipicephalus (Boophilus) microplus* larvae in the state of São Paulo

Samples	RF	Genotypes			
			SS	RS	RR
		n	% freq.	% freq.	% freq.
P10	10.3	16	0	87.5	12.5
P11	6.85	11	54.5	27.3	18.2
P14	4.89	16	93.75	6.25	0
P15	3.39	16	93.75	6.25	0
P16	4.63	16	93.75	0	6.25
P19	28.2	13	23.1	15.4	61.5
P20	8.00	16	100	0	0
P27	5.94	16	93.75	6.25	0
P36	14.7	16	100	0	0
P37	8.27	16	100	0	0

N = number of individuals; LC_{50} (95% CI) = concentration that kills 50%, with confidence interval; RF = resistance factor; RL = resistance level



S: Sensitive; R: Resistant; 7-13: Sample Number; C + S: sensitive positive control; C + R: resistant positive control.

Figure 3. PCR amplification of 13 samples from the P19 farm. The picture shows the results of samples 7 through 13 amplified by the primer pair specific for the sensitive (227I + 221S) or resistant allele (227I + 221R).

DISCUSSION

Use of chemicals against *R. microplus* has been considered the most effective and economical way to control it for decades. However, continuous and uncontrolled use of acaricides has led to emergence of strains that are resistant to most of the compounds available on the market, according to Higa *et al*. (2016).

In general, development of pest resistance to insecticides depends on the quantity and frequency of their application. In the present study, reports by the owners regarding the commercial products used revealed that most of the producers were using acaricides composed of associations of organophosphates and pyrethroids. Among these farms, 89% showed resistance to diazinon and 11% to cypermethrin. The low frequency of resistance to cypermethrin was probably related to the fact that it was used together with organophosphates in the same product, thus suggesting that organophosphate was more effective than pyrethroids when used in combination.

As shown in the results from the present study, resistance of *R. microplus* to cypermethrin was found in 18 of the 40 farms analyzed but it was seen that the resistance values remained low. The hypothesis of high resistance to pyrethroids in this study was discarded through the unexpected results of high susceptibility among the samples analyzed.

The resistance to acaricides observed in the present study contradicts data reported by Higa *et al*. (2016) and Albuquerque *et al*. (2010). Those authors found that *R. microplus* strains in the state of São Paulo were resistant to products composed only of pyrethroids and thus showed that pyrethroids had low efficacy in field populations. Lovis *et al*. (2013) showed that there were high levels of resistance to cypermethrin in the state of São Paulo, with resistance ratio ranging from 8.0 to 309.3. Their values are discordant with our results. Klafke *et al*. (2017) evaluated *R. microplus* samples in Rio Grande do Sul using a diagnostic dose and found that resistance to cypermethrin was present in 98.08% of the 104 samples analyzed.

The results suggest that pyrethroids have reasonable efficacy against *R. microplus* strains in the state of São Paulo State. One hypothesis to be considered is that the quality control on the commercial products tested may have failed. Products may also have been inadequately stored on these farms. The test on technical cypermethrin in this study caused high mortality among the larvae.

The site of action of pyrethroids is the sodium channel of arthropods, and five mutations associated with resistance were described in detail by Kumar *et al*. (2020). Five other non-synonymous mutations were also found, but without any safe correlation in relation to the pyrethroid-resistant phenotype. Among these mutations, the only one reported so far in Brazil was C190A (domain II S4-5), notified by

Andreotti *et al*. (2011), Domingues *et al*. (2012) and Lovis *et al*. 2013. In the present study, seven *samples of R microplus* (resistance factor from 3.39 to 28.2) were found to have mutation T2134A in domain III S6 (F1550I: replacement of a phenylalanine by an isoleucine).

This is the first report of this mutation in Brazil. It has previously been cited in tick strains that were highly resistant to pyrethroids, in Mexico and the United States (Rodríguez-Vivas *et al*., 2014; Stone *et al*., 2014; Kumar *et al*., 2020). The absence of a direct correlation between resistant phenotypes and the genotypes found can be explained by the presence of other mutations for which no detection tests have been performed. The results from the present study open up two possibilities: either new trials should be implemented or questioning about the influence of other factors arises.

The phenotypes found in the larval packet test results not only may be explained by the T2134A mutation, but also may be consequences of four other mutations that have already been described as associated with resistance to pyrethroids, as reported by Kumar *et al*. (2020). Consideration should also be given to the metabolic capacity of R. *microplus* relating to phenotype variations. Occurrences of sensitive genotypes with resistant phenotypes, which would suggest that catalytic sites of certain enzymes may have higher metabolic detoxification capabilities, were reported in studies by Cossio-Bayugar *et al*. (2009) and Miranda *et al*. (2009).

Several studies have reported resistance to organophosphates in *R. microplus* in Brazil, as described by Mendes *et al*. (2011), Domingues *et al*. (2012), Raynal *et al*. (2013) and Reck *et al*. (2014). The results obtained for diazinon in phenotypic tests corroborated the findings of these previous studies. Thus, it can be affirmed that the situation of resistance to organophosphates oscillates among farms is a consequence of their history of use of acaricides. Animal treatment failures also contribute to this situation (Lovis *et al*., 2013)

Regarding the activity of esterase against live and dead larvae, it was evident that the P19 sample, which was the one with the highest resistance factor (28.2) for cypermethrin, showed the highest esterase activity level. This confirms what was reported by Lovis *et al*. (2013), i.e., that this activity in ticks is often related to development of resistance to pyrethroids. In the enzymatic profiles detected for the larval samples tested in this study with diazinon, it was observed that increased α- and β-esterase levels in live larvae showed a positive correlation with resistance factors. Similarly, the metabolic relationship of the enzyme in the resistance process was demonstrated by Miller *et al*. (2008) and Chigure *et al*. (2017). The latter authors confirmed that there was a positive correlation between higher enzymatic activity of esterase and occurrences of resistance to diazinon in *R. microplus* samples in Mexico.

This paper presents the first report of the T2134A mutation (F1550I) in a *R. microplus* population in Brazil. These resistance data regarding cypermethrin and diazinon form useful information for future decision-making, considering the frequent use of acaricide products based on organophosphate compounds and their associations in different regions of the state of São Paulo.

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