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A new approach to characterization of the resistance of populations of *Rhipicephalus microplus* (Acari: Ixodidae) to organophosphate and pyrethroid in the state of Minas Gerais, Brazil

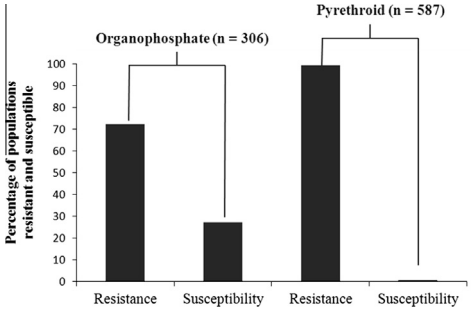
Aline Pasqualini Faza [a,*], Isabella Silvestre Barreto Pinto [b], Isabela Fonseca [c], Gustavo Resende Antunes [b], Caio Márcio de Oliveira Monteiro [d], Erik Daemon [a], Michelle de Souza Muniz [b], Marta Fonseca Martins [b], John Furlong [b], Márcia Cristina de Azevedo Prata [b]

[a] *Programa de Pós-graduação em Biologia e Comportamento Animal da Universidade Federal de Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil*
[b] *Embrapa Gado de Leite, Rua Eugênio do Nascimento, 610 Bairro Dom Bosco, 36038-330 Juiz de Fora, Minas Gerais, Brazil*
[c] *Programa de Pós-graduação em Genética e Melhoramento da Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil*
[d] *Programa de Pós-graduação em Ciências Veterinárias da Universidade Federal Rural do Rio de Janeiro, Rio de Janeiro, Seropédica, Brazil*

HIGHLIGHTS

- Of the 587 populations tested for pyrethroids, 97.44% were resistant.
- For organophosphates were tested 306 populations and 75.49% were resistant.
- Into populations resistant to pyrethroids, 91% are heterozygous.
- The analysis confirmed the serious problem of resistance of *Rhipicephalus microplus* populations.

GRAPHICAL ABSTRACT



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ABSTRACT

The monitoring of resistance of cattle tick populations in Brazil to the chemical bases in use is largely limited to investigation of the phenotypic profile. There are few studies investigating the role played by the genotypic profile in acaricide resistance in the country. Therefore, the aim of the present study was to carry out molecular characterization and trace out the genetic profile of populations of *Rhipicephalus microplus* with respect to resistance to the organophosphate and pyrethroid chemical groups. For that purpose, larvae were genotyped belonging to 587 populations for pyrethroids and 306 for organophosphates, using the polymerase chain reaction technique. It was found that 75.49% and 97.44% of the larvae studied showed resistance to the organophosphates and pyrethroids, respectively. Among the populations resistant to pyrethroids, 91.9% were heterozygotes, showing that most of the resistant populations have only one allele responsible for resistance. Therefore, it is possible to conclude that the genotyped populations have high resistance to organophosphates, and even more so to pyrethroids. This information is fundamental for understanding the mechanisms of resistance of *R. microplus* to acaricides, to enable improvement of control techniques.

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1. Introduction

Rhipicephalus microplus (Canestrini, 1888) (Acari: Ixodidae) is a cattle ectoparasite of great concern in tropical and subtropical countries because it causes blood spoliation and transmits pathogens, significantly lowering beef and milk production and

* Corresponding author.
E-mail addresses: alinefaza@yahoo.com.br (A.P. Faza), isabellajf@gmail.com (I.S.B. Pinto), isabela_fonseca@yahoo.com.br (I. Fonseca), guto1982@gmail.com (G.R. Antunes), caiosat@gmail.com (Caio Márcio de Oliveira Monteiro), erik@aces-sa.com (E. Daemon), mmuniz@cnpgl.embrapa.br (Michelle de Souza Muniz), mmartins@cnpgl.embrapa.br (M.F. Martins), john@cnpgl.embrapa.br (J. Furlong), mprata@cnpgl.embrapa.br (Márcia Cristina de Azevedo Prata).

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increasing herd management costs. According to data from the Ministry of Agriculture, this cattle tick causes losses of some two billion dollars a year in Brazil (Grisi et al., 2002).

While alternative strategies such as the use of vaccines, phytotherepeutics and biological control agents are under intense study and improvement, the control of this arthropod is still mainly done with chemical compounds (George, 2000). However, the results often fall short of expectations because the repeated and often inadequate use of these compounds leads to the selection of resistant tick (Gomes et al., 2011). Records of populations resistant to pyrethroids and organophosphates have been made in several regions of the world, and more recently also have been recorded populations resistant to amitraz, macrocyclic lactones, fipronil (Martins and Furlong, 2001; Li et al., 2004; Klafke et al., 2006; Klafke 2008; Castro-Janer et al., 2010a,b). Because of the problem with resistance, in 1997 the Embrapa Dairy Cattle Research Unit (Embrapa Gado de Leite) implemented a program to test the efficacy of acaricides, to determine the most adequate products to apply in each farm by analyzing specimens sent by producers (Furlong et al., 2007). This bioassay is based on immersion of engorged females (Drummond et al., 1973) and supplies phenotypic information on the level of resistance of different tick populations to the most common chemical acaricide compounds (Furlong et al., 2007), but without elucidating the genotypic origins of that resistance.

Currently, evaluating resistance has also been made using molecular techniques that allow determine the allelic frequency and realize genotypic characterization of the populations, beyond to provide diagnosis in a short period (less than the time required for performing bioassays) (Klafke, 2008). Recently, the use of molecular techniques have provided information about the resistance of *R. microplus* to pyrethroids and organophosphates at different locations in the world (Guerrero et al., 2001, 2002; Hernandez et al., 2002; Pereira et al., 2004; Baffi et al., 2007; Cruz et al., 2009).

In line with these efforts, the aim of the present study was to add further information on the resistance phenomenon, by tracing out a genotypic resistance profile of cattle tick populations to organophosphates and pyrethroids.

2. Material and methods

The *R. microplus* larvae used in this study were obtained from the populations in the state of Minas Gerais, being utilized larvae of the control group of each immersion test (Drummond et al., 1973) performed between 2005 and 2009 at the Parasitology Laboratory of Embrapa Gado de Leite, in Juiz de Fora, Minas Gerais, from specimens sent by farmers. These larvae were placed in previously labeled microtubes and taken to the Molecular Genetics Laboratory (Dr. Mário Luiz Martinez) for genotypic characterization of the resistance to organophosphates and pyrethroids. For that purpose, were conducted 893 tests, being genotyped 587 populations for pyrethroids and 306 for organophosphates, using the polymerase chain reaction technique. All populations were tested for both organophosphate as for pyrethroid, and the difference between the numbers of samples tested for each acaricide was due to does not amplification in some tests.

2.1. Extraction of DNA from the larvae

The DNA from the larvae was extracted according to Sheppard and Hinkley (1992) with some modifications. One larva from each population was placed in a microtube and macerated in 300 μl of grinding buffer (10 mM Tris–HCl, 60 mM NaCl, 30 mM sucrose, 10 mM EDTA). Then 300 μl of lysis buffer (300 mM Tris–HCl, 40 mM SDS, 20 mM EDTA) was added and the samples were incubated on ice for 15 min. After that, 5 μl of proteinase K (20 μg/μl) was added and the samples were incubated at 56 °C for 1 h. Then, 300 μl of phenol and 300 μl of chloroform: isoamyl (24:1) were added and the samples were mixed by pipette and centrifuged for 5 min at 17,000×*g*.

The upper phase was transferred to a new microtube and 600 μl of chloroform: isoamyl (24:1) was added and the samples were once again mixed by pipette. Then the samples were centrifuged for 5 min at 17,000×*g* and the upper phase was transferred to another microtube. Next, 60 μl of NaCl 5 M and 1000 μl of ethanol were added for precipitation of the DNA and the samples were stored at −20 °C overnight. The next step was to wash the resulting pellet with 100 μL of 70% ethanol and centrifuge the microtube at 17,000×*g* for 5 min. The supernatant was then discarded and the tubes were left open on their side on a sterile surface for 30 min at room temperature. The last step was to resuspend the pellet in 20 μl of ultrapure water.

After the extraction, the quantification and quality evaluation of the samples were carried out by means of spectrophotometry (NanoDrop®1000, Thermo Fisher Scientific, Wilmington, DE, USA). The parameters to assess the sample quality were concentration (ng/μL) and purity, called $A_{260/280,}$ whose ideal value is 1.8. The proof of this methodology was accomplished by the polymerase chain reaction (PCR) technique.

2.2. PCR and electrophoresis for molecular characterization

For the PCR a test was performed to verify the optimal amplification condition of each marker, by determining the annealing temperature and magnesium concentration. The samples were amplified in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA, USA), according to the specific conditions of the pairs of primers utilized to evaluate the resistance to pyrethroids and organophosphates.

2.3. PCR for organophosphates

To determine the resistance and susceptibility of the larvae to the organophosphate chemical group, the primer pair GS138B 5′-GCA TCGACCTCTCGTCCAAC-3′ and GS139R 5′-GTCGGCATACTTGTCTTCG ATG-3′ was used, as described by Hernandez et al. (2002).

The PC reaction was carried out with 20 ng of DNA, 0.5 μM of each primer and 1X of GoTaq®Green Master Mix (Promega) in a final volume of 25 μL. The PCR consisted of a prior denaturing at 95 °C for 5 min, followed by 10 cycles at 95 °C for 1 min, 65 °C for 1 min (with decrease of 1 °C per cycle) and 72 °C for 1 min, followed by 30 cycles at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 7 min.

The PCR product was digested with the restriction enzyme EcoRI at 37 °C for 3 h and submitted to electrophoresis in 1.8% agarose gel, stained with 0.001% ethidium bromide. As before, the gel was revealed with an Eagle Eye II Imaging System (Stratagene) to identify the bands.

2.4. PCR for pyrethroids

To obtain the genotype for resistance and susceptibility of the cattle tick larvae to the pyrethroid chemical group, two forward primers were employed: FG222 5′-TTATCTTCGGCTCCTTCA-3′ and FG221 5′-TTATCTTCGGCTCCTTCT-3′, along with a reverse primer common for the two: FG227 5′-TTGTTCATTGAAATTGTCGA-3′, as described by Guerrero et al. (2001). For the PCR, 20 ng of DNA, 1.0 μM of each primer and 1X of GoTaq®Green Master Mix (Promega, Madison, WI, USA) were used, in a final volume of 25 μL. The reaction consisted of prior denaturing at 95 °C for 5 min, followed

by 10 cycles with denaturing at 95 °C for 1 min, annealing at 65 °C for 1 min (with a decrease of 1 °C per cycle) and extension at 72 °C for 1 min, followed by 30 cycles with denaturing at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and final extension at 72 °C for 7 min.

Then the samples were submitted to electrophoresis in 3% agarose gel, stained with 0.001% ethidium bromide. The gel was revealed with an Eagle Eye II Imaging System (Stratagene, La Jolla, CA, USA) to identify the bands.

2.5. Statistical analysis

The values obtained in percentage were transformed into arcsine x. The means of the percentage of resistance and susceptibility to organophosphates and pyrethroids were compared by ANOVA and the Tukey test at 5% significance.

3. Results and discussion

In the gel presented in Fig. 1, three bands can be identified, referring to the alleles related to resistance to organophosphates: one fragment of 372 base pairs (bp), which characterizes allele A (sensitive), two fragments, of 300 bp and 72 bp, which determine allele B (resistant), and three fragments, of 372, 300 and 72 bp, which characterize genotype AB (moderate resistance).

The results obtained show significant differences ($p < 0.05$) between the average percentage of larvae susceptible (27.3%) and resistant (72.2%) to organophosphates (Table 1), which can either be homo or heterozygotes for the gene that codifies *CaE*. The analysis of these results indicates the situation is more serious than shown by previous studies, because the profile determined by the phenotypic analyses of these same populations evidenced levels of 40.66% and 59.34% for resistance and susceptibility to organophosphates, respectively. A similar situation was found by Baffi et al. (2007) with cattle tick populations from the municipality of Uberlândia in Minas Gerais, with genotypic profiles of 23% and 77% and corresponding phenotypic profiles of 50.77% and 49.33% for sensitivity and resistance to organophosphates, respectively. The explanation for the apparent discrepancy between the genotypic and phenotypic profiles in the two experiments can be related to the semidominance phenomenon. According to Klafke (2008), resistance to organophosphates is normally determined by a single semidominant gene. As a consequence, heterozygote individuals manifest resistance, but to a lesser degree than resistant homozygotes. These differences can also be due to the occurrence of mutations at different sites than those evaluated in the respective experiments. In any case, the results presented here confirm the intense selective pressure in favor of resistance to organophosphates, one of the oldest chemical groups used to control *R. microplus* in Brazil and one of the most commonly used in the composition of acaricides available at present, whether in single formulations or associated with other chemical groups (Gomes et al., 2011).

The 68 bp fragment amplified with the FG221/FG227 primer pair characterizes susceptibility (allele A) to pyrethroids and the 68 bp fragment amplified with the FG222/FG227 primer pair characterizes resistance (allele B). The presence of the two fragments (AB) indicates moderate resistance (Fig. 2) of the larvae to this chemical compound.

There were also significant differences ($p < 0.05$) between the average percentage of larvae resistant (99.1%) (AB or BB) and susceptible (0.9%) (AA) to pyrethroids (Table 1). In this case, 91.9% of the resistant larvae were identified as heterozygotes (AB) and 5.54% as homozygotes (BB). These results indicate that the majority of the populations resistant to pyrethroids have only one allele responsible for this resistance. This information is extremely important because it means it may be possible to reverse the resistance to this chemical group, for example by introducing sensitive individuals in the population, thus stimulating studies to make this practice possible.

In the case of pyrethroids, the comparison between the genotypic and phenotypic results showed that the latter had a 100% resistance profile, demonstrating correspondence of results and confirming the serious situation of resistance. In practice this means that single pyrethroid formulations are totally ineffective in controlling the cattle tick populations studied. The same finding was reported by Gomes et al. (2011). The high-resistance

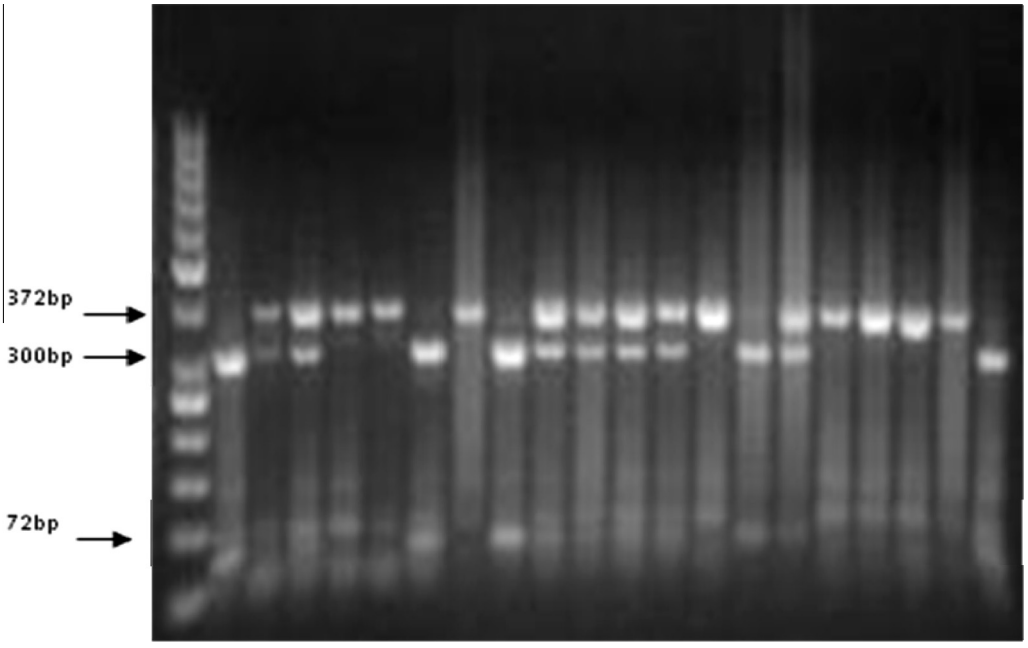

Fig. 1. Products of PCR after digestion with restriction enzyme *EcoRI*, in larvae of *Rhipicephalus microplus* applied in agarose gel stained with ethidium bromide showing the three fragments 372, 300 and 72 base pairs (bp) which confer resistance/susceptibility to chemical group of organophosphate.

Table 1
Number of populations of *Rhipicephalus microplus* genotyped and percentage of resistance/susceptibility per year for pyrethroid and organophosphate.

Chemical bases	Organophosphate				Pyrethroid			
Ano	Resistance		Susceptibility		Resistance		Susceptibility	
	N	%	*N*	%	*N*	%	*N*	%
2005	61	80.3	15	19.7	63	100.0	0	0.0
2006	12	75.0	12	25.0	...	...	...	...
2007	101	74.3	35	25.7	46	100.0	0	0.0
2008	10	55.6	8	44.4	38	100.0	0	0.0
2009	47	78.3	13	21.7	425	96.6	15	3.4
Mean (%) ± S.D		72.2^{a} ± 8.8		27.3^{b} ± 8.8		99.1^{a} ± 1.3		0.9^{b} ± 1.5

Means were compared by ANOVA and Tukey test at a significance level of 5%. Different letters indicate significant difference between the percentage of susceptibility and resistance in the chemical group.
Means followed by letters different differ significantly (P<0.05). So, letters "a" and "b" indicates so differences.
N – Number of populations.
... – Tests not performed.

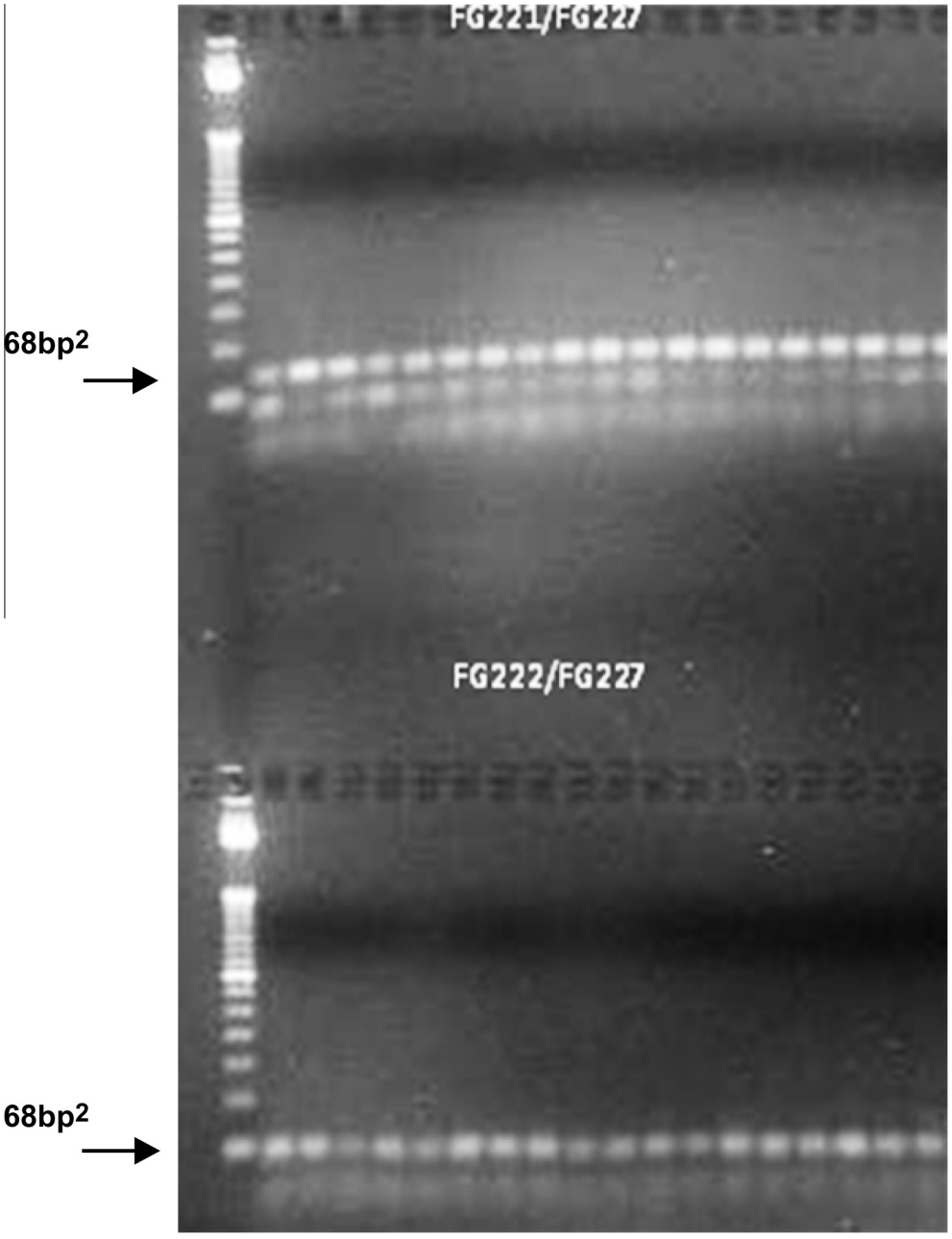

Fig. 2. Products of PCR after digestion with restriction enzyme *EcoRI*, in larvae of *Rhipicephalus microplus* applied to agarose gel stained with ethidium bromide showing the two 68^{1} base pairs (bp) fragments which confer susceptibility and 68^{2} bp fragment that confers resistance to the chemical group of pyrethroids.

phenotypic profile to this chemical group was also observed by Mendes et al. (2011), according to whom there was 82.6% resistance to cypermetrin and 86.36% to deltametrin. Besides this, 50% of the 24 populations tested by those authors showed resistance to the two groups studied, pyrethroids and organophosphates, demonstrating generalized resistance to the available chemical bases. In studies conducted in other regions, although high resistance to pyrethroids was reported (Guerrero et al., 2002; Hernandez et al., 2002), the situation was not as extreme as diagnosed in Brazilian cattle tick populations.

In the study carried out by Guerrero et al. (2001), 11 populations were analyzed from Mexico and Texas. Of these, only four were considered to be resistant to pyrethroids, unlike found in this study, in which the majority of the genotyped larvae were diagnosed as resistant. This difference may have occurred due to the complexity of development of this resistance, because it involves various enzymes and metabolic pathways. It is thus probable that various other genes play important roles in the overall resistance profile. Besides this, the populations come from different countries with different herd management practices. The resistance profile found in Brazil, especially in the state of Minas Gerais, is reason for great concern, making control much harder.

According to Mendes et al. (2011), the main factors leading to increased resistance are associated with the choice of the acaricide product. This choice was made according to recommendations by 85.8% of the farmers surveyed in that study, while 10.8% did not have any particular criterion and only 3.4% relied on the result of immersion tests.

The results obtained in the present study are an warning of the need to establish programs to orient producers regarding the rational use of the available acaricides, to achieve efficient control and delay the development of resistance, thus preserving the effectiveness of the few chemical bases available, to assure better health of the animals and consequently of consumers of the products obtained from them. Proper choice and use of acaricides will also help reduce production costs and risks of environmental contamination.

Finally, the results here indicate the need to carry out studies of techniques to reverse resistance to pyrethroids and to shed more light on the processes related to resistance to organophosphates, such as the semidominance phenomenon.

4. Conclusion

The genotypic analysis confirmed the serious problem of resistance of cattle tick populations in the state of Minas Gerais, Brazil to the principal chemical bases of acaricides, especially pyrethroids.

Acknowledgments

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