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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

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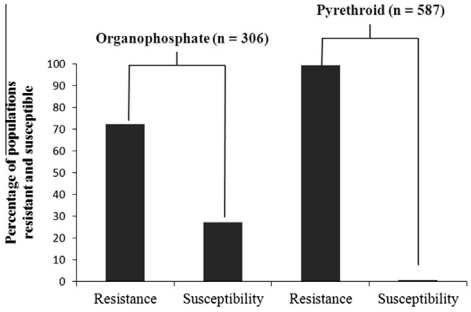
HIGHLIGHTS

- 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.

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

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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