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Survey of pyrethroid and organophosphate resistance in Brazilian field populations of *Rhipicephalus* (*Boophilus*) *microplus*: Detection of C190A mutation in domain II of the *para*-type sodium channel gene

Luísa Nogueira Domingues[a,*], Bruno dos Santos Alves Figueiredo Brasil[a,b], Ana Cristina Passos de Paiva Bello[a], Arildo Pinto da Cunha[a], Antonio Thadeu Medeiros de Barros[c], Romário Cerqueira Leite[a], Cornelia Silaghi[d], Kurt Pfister[d], Lygia Maria Friche Passos[a,d]

[a] *Escola de Veterinária, INCT, Informação Genético Sanitária da Pecuária Brasileira, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, CP 567, CEP 31270-901, Belo Horizonte, MG, Brazil*

[b] *Valid Biotechnology Research Team, INOVA, Universidade Federal de Minas Gerais, Belo Horizonte, MG, 9 Brazil*

[c] *Empresa Brasileira de Pesquisa Agropecuária (Embrapa Pantanal), Rua 21 de Setembro, 1880, Bairro Nossa Senhora de Fátima, CP 109, CEP 79320-900, Corumbá, MS, Brazil*

[d] *Lehrstuhl für Vergleichende Tropenmedizin und Parasitologie, Ludwig-Maximilians-Universität München, Leopoldstr. 5, 80802 München, Germany*

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ABSTRACT

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* causes expressive damage to livestock in Brazil and other countries. Its control is becoming more difficult due to the development of resistance in populations. Early detection of resistance can help in developing effective control strategies. This study evaluated the susceptibility of *R. microplus* to cypermethrin and chlorpyriphos and was the first attempt to identify the mechanism of resistance (target site insensitivity) in cattle tick populations from Minas Gerais state (Southeastern Brazil). Engorged female ticks were collected from 10 ranches within the state of Minas Gerais, and susceptibility was evaluated with the larval packet test (LPT) using technical grade cypermethrin and chlorpyriphos. It was possible to analyze LPT results of seven populations. Target site insensitivity was investigated in all 10 isolates by using molecular approaches for detection of the T2134A substitution within the domain III S6 segment and the C190A in the domain II S4-5 linker from the *para*-type sodium channel gene. LPT showed that all seven populations were resistant to cypermethrin with resistance ratio (RR) ranging from 16.0 to 25.0 and 85.7% were resistant to chlorpyriphos (RR = 2.2–15.6). Although the T2134A mutation was not detected, the C190A mutation was highly prevalent, being present in 82–100% of the alleles sampled in field populations. A significant correlation was found between the LC50 values for cypermethrin and the frequency of the C190A mutation suggesting that it might be responsible for the phenotypic resistance detected.

1. Introduction

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* (Canestrini, 1887) (Acari: Ixodidae) is one of the most important parasites of cattle in tropical and subtropical countries. In Brazil, it is responsible for annual losses of about U$2 billion due to mortality, decrease in both milk production and weight gain, deteriorating effects on leather quality, costs for acaricide drugs and transmission of cattle fever disease agents (Grisi et al., 2002).

The control of *R. microplus* mainly relies on the use of chemical products mostly without following any

* Corresponding author. Tel.: +55 31 3409 2098; fax: +55 31 3409 2080.
E-mail address: dominguesln@gmail.com (L. Nogueira Domingues).

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technical criteria (leading to an excessive number of applications and too low volume of product per animal) which contributes to accelerating the development of resistance to acaricides (Alonso-Díaz et al., 2006; Mendes et al., 2007, 2011). In Brazil, the first record of cattle tick resistance to organophosphates and pyrethroids was in the 1970s and 1980s, respectively (Arteche, 1972; Leite, 1991). Resistance persisted and now it is found throughout the country (Alonso-Díaz et al., 2006; Andreotti et al., 2011; Mendes et al., 2011).

Pyrethroids exert a neurotoxic effect on arthropods by binding to the sodium channels and prolonging the opening of these transmembrane proteins and inhibiting the deactivation and stabilizing the open configuration of the channel (Dong, 2007). Structural changes in sodium channels due to mutations may decrease the interaction between pyrethroids and its target site, and thus reduce the sensitivity of arthropods to these acaricides (Dong, 2007). Three mutations in the sodium channel have been associated with resistance to pyrethroids in *R. microplus* populations (He et al., 1999; Chen et al., 2009; Morgan et al., 2009; Jonsson et al., 2010; Guerrero et al., 2012).

He et al. (1999) identified a point mutation in the S6 segment of domain III of the *para*-type sodium channel of Mexican strains of *R. microplus* resistant to permethrin. This mutation involves the substitution of a thymine by an adenine (T2134A), resulting in the replacement of a phenylalanine by an isoleucine at susceptible and resistant individuals, respectively.

The mutation described by Morgan et al. (2009) is located at domain II S4-5 linker of the *para*-sodium channel gene and it is a substitution of a cytosine in the susceptible strain to an adenine in the resistant strain (C190A). This substitution led to a leucine to isoleucine replacement that was correlated to pyrethroid resistance (Morgan et al., 2009). Jonsson et al. (2010) reported another substitution in tick populations from Australia: G214T in the domain II S4-S5 linker, which is a glycine to valine change that is associated with resistance to the pyrethroid flumethrin only.

Both detection of the levels of acaricide resistance and understanding the mechanism of resistance in *R. microplus* are important to the development of an effective tick control program. A rational use of pesticides will help to delay the development of resistance and reduce pesticide contamination of the environment as well as chemical residues in meat and milk. This study aimed at evaluating (i) the susceptibility of Brazilian field populations of *R. microplus* to the synthetic pyrethroid cypermethrin and the organophosphate chlorpyriphos and (ii) the role of target site insensitivity mediated by T2134A and C190A substitutions.

2. Materials and methods

2.1. Tick collection

In April 2010, 100 engorged females of *R. microplus* were collected from 10 cattle ranches in the 'Triângulo Mineiro' and 'Alto Paranaíba' regions within the state of Minas Gerais in Southeastern Brazil. The state has the highest milk production in the country and is a leading producer of beef cattle (Pesquisa, 2009). After collection, ticks were stored in plastic containers and sent by post to the Laboratory of Parasitic Diseases, School of Veterinary Medicine, Federal University of Minas Gerais, Belo Horizonte.

2.2. Larval packet test

The bioassay, larval packet test (LPT) (Stone and Haydock, 1962), recommended by FAO (2004), was conducted to detect resistance to cypermethrin and chlorpyriphos.

Ticks were washed with distilled water, dried on absorbent paper and divided into Petri dishes, 30 ticks per plate (separated according to the ranch). These dishes were kept in an incubator at $28 \pm 1.5\ ^{\circ}C$ and approximately 85% relative humidity and 14 days later, eggs were collected and transferred to glass tubes sealed with hydrophobic cotton to allow larval hatching. Egg masses from many female ticks from the same farm were mixed before hatching so that larvae used in these experiments were not all siblings.

Technical grade cypermethrin (93.59% purity) (Allvet®, Londrina, Brazil) was serially diluted in a mixture of trichloroethylene (Synth, Diadema, Brazil) and olive oil (Sigma–Aldrich, São Paulo, Brazil) (2:1, v/v), resulting in different concentrations (in % of active ingredient): 5, 4, 2.4, 2.04, 1.632, 0.979, 0.588, 0.353, 0.212, 0.127 for field populations and 0.1, 0.06, 0.022, 0.013, 0.008, 0.005, 0.003, 0.002 for the *R. microplus* 'Porto Alegre' strain. This strain has been maintained at the Instituto Biológico de São Paulo without contact to acaricides and is considered susceptible.

Filter papers (Whatman n° 1) measuring 8.5 cm × 7.5 cm were impregnated with 0.67 ml of each cypermethrin concentration, including the negative control (only the mixture of trichloroethylene and olive oil). Two papers were used per concentration. Approximately 100 larvae, aged between 14 and 21 days, were added to each of these papers which were folded and sealed with bulldog clips on the sides and top. Papers were stored in the incubator under the conditions described above and larvae mortality was assessed after 24 h of exposure. Larvae unable to move were considered dead.

The same dilution and larvae exposure procedures were performed with chlorpyriphos (97.43% purity) (Ourofino, Cravinhos, Brazil). In this case the concentrations used were (in % of active ingredient): 0.128, 0.064, 0.032, 0.016, 0.008, 0.004, 0.002, 0.001, 0.0005, and 0.00025 for both field populations and 'Porto Alegre' strain.

Mortality data were analyzed by POLO-PC (Leora Software, 1987) in order to obtain the lethal concentration for 50% of the population (LC_{50}) with a 95% confidence interval (CI 95%). The resistance ratio (RR) was calculated by dividing the LC_{50} obtained from the field populations by the LC_{50} obtained from the 'Porto Alegre' susceptible reference strain. Differences in LC_{50} were considered significant when their 95% fiducial limits did not overlap. Tests showing mortality rates between 5% and 10% in the control group were submitted to Abbott's formula (Abbott, 1925).

2.3. Detection of T2134A mutation

Larvae that were not used in LPT were stored in 99% ethanol and kept at −20 °C for later molecular analysis. DNA was purified from individual larvae with the Qiagen DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions for animal tissue. Larvae were incubated overnight at 56 °C with proteinase K to allow thorough dissolving of the tissue. Each specimen (30 per cattle ranch, 300 in total) was genotyped for the presence of the T2134A substitution in the S6 trans-membrane segment of domain III of the *para*-type sodium channel described by He et al. (1999) using an allele-specific PCR as reported by Guerrero et al. (2001).

In each PCR reaction (total volume 50 μl) 5 μl of template DNA, 0.5 μl of each primer (FG227 and FG221 or FG227 and FG222; 100 μM) (Eurofins MWG/Operon) were included combined with 5 μl Puffer 10×, 1 μl dNTPs (10 mM), 0.25 μl Taq Polymerase (5 U/μl), 2 μl $MgCl_2$ (2.5 μM) and 35.75 μl molecular grade water. The HotStart Taq Plus Polymerase Kit (Qiagen, Hilden, Germany) was used for the PCR reactions. Amplifications were carried out using a ABVeriti thermocycler (Applied Biosystems, Darmstadt, Germany) programmed for 95 °C for 5 min, 37 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 7 min. PCR products were fractioned on 2% agarose gel including GelRed™ (Biotium, Hayward, USA) with a 50 bp ladder (Fermentas, St. Leon-Rot, Germany) and made visible under UV light.Larvae DNA yielding an amplicon of 68 bp only at the reaction with primers FG227 and FG221 were considered homozygous susceptible (SS). Larvae DNA yielding an amplicon (68 bp) only at the reaction with primers or FG227 and FG222 were considered homozygous resistant (RR). Larvae DNA yielding amplification in both reactions were considered heterozygous (SR).

A pool of larvae of 'San Felipe' strain (courtesy of Dr. Felix Guerrero) was used as control to both alleles and molecular grade water was used as blank. The 'San Felipe' strain has been maintained under selection pressure with the pyrethroid permethrin for several generations at the USDA-ARS Cattle Fever Tick Research Laboratory (CFTRL) in Mission, TX, USA. This strain has specimens with both susceptible and resistant alleles, although the latters are present at much higher frequency (F Guerrero personal communication).

2.4. Detection of C190A mutation

Genomic DNA was purified from individual larvae (~30 larvae of each ranch) as described by Guerrero et al. (2001) with the following modifications. Larvae that were stored at ethanol were washed in distilled water, transferred to 1.5 ml micro centrifuge tubes and placed in liquid nitrogen. A plastic pestle for 1.5 ml centrifuge tubes was used to crush and grind the larva against the tube wall, until close visual inspection ensured that the larva was broken into several fragments. Twenty five microliters of sample buffer (Tris–HCl 1 M, pH 7.5; KCl 1 M; pure water) were added to the tube and after all larvae were prepared, the tube contents were mixed and centrifuged during 20 s to ensure that the liquid and crushed larva were at the tube bottom. The tubes were moved back to liquid nitrogen and then placed in a boiling water bath for 5 min. Finally, the tubes were transferred back to the liquid nitrogen and then were stored at −20 °C.

A 25 μL PCR was performed with 13.25 μL of ultrapure water, 5 μL of GoTaq 5X PCR buffer (2.5 mM $MgCl_2$) (Promega, USA), 0.5 μL of each primer (10 μM), 2.5 μL of each dNTP (1 mM), 0.25 μL of GoTaq DNA polymerase (Promega) (5 U/μL) and 3.0 μl of DNA template (50–100 ng/μL). PCR conditions in an automated thermocycler (Veriti-Life Technologies, USA) were the following: initial denaturation at 95 °C for 1 min, 50 °C for 45 s and 72 °C for 90 s, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 90 s, with a final elongation step at 72 °C for 7 min. It was used the same primers described by Morgan et al. (2009): BmNaF5 5′-TACGTGTGTTCAAGCTAGC-3′ and BmNaR5 5′-ACTTTCTTCGTAGTTCTTGC-3′.

PCR products (5 μL) were visualized on agarose gels and selected for direct sequencing. Sequences were determined bi-directionally using the BigDye Terminator v.3.1 Cycle Sequencing Kit on the automated DNA sequencer ABI 3130 (both from Life Technologies, USA), in accordance with the manufacturer's instructions. Forward and reverse sequences were aligned and edited using SeqScape software® (Life Technologies) and genotyped based on the presence of the C190A mutation.

3. Results and discussion

Populations of *R. microplus* resistant to different active ingredients are present in almost all countries where these parasites occur (Alonso-Díaz et al., 2006). In Brazil the situation is not different: several studies have shown that populations of this parasite are resistant to almost all available drugs including macrocyclic lactones and phenylpyrazole (Arteche, 1972; Leite, 1991; Klafke et al., 2006; Mendes et al., 2007, 2011; Castro-Janer et al., 2010; Andreotti et al., 2011).

Seven populations surveyed by LPT showed RR between 16.0 and 25.0 to cypermethrin (Table 1) and between 2.2 and 15.6 to chlorpyriphos (Table 2). All these populations can be considered resistant level II to cypermethrin while one population can be considered susceptible, two populations resistant level I and four populations resistant level II to chlorpyriphos, according to a classification described by Mendes et al. (2007). It was not possible to calculate the LC_{50} and its CI 95% of three populations because the control group had mortality higher than 10%. Unfortunately it was not possible to repeat these tests.

Nolan et al. (1989) demonstrated that cyhalothrin (0.007%) applied to animals to control *R. microplus* infestations had an efficacy of 90.2% against Marmor strain (RR = 6 to cypermethrin) and 33.4% against Parkhurst strain (RR = 114 to cypermethrin). Considering this data, synthetic pyrethroids will probably not be effective to control the cattle tick at the ranches included in this study as the surveyed populations had a RR almost two times higher than the Marmor strain (Table 1). The same situation can occur with organophosphates (Table 2), according to Patarroyo

Table 1
Susceptibility of *R. microplus* to cypermethrin in the 'Triângulo Mineiro' and 'Alto Paranaíba' regions within the state of Minas Gerais, Southeast Brazil, April 2010.

Cattle ranches	City	Cypermethrin			
		n	χ^2 (df)	LC_{50} (CI 95%)	RR
1	Uberlândia	1134	6.56 (4)	0.48 (0.43–0.53)	16.0
2	Uberlândia	862	11.31 (6)	0.56 (0.45–0.67)	18.7
3	Uberlândia	1725	13.25 (10)	0.56 (0.51–0.62)	18.7
4	Uberlândia	1485	15.63 (10)	0.69 (0.63–0.75)	23.0
5	Uberlândia	1440	11.86 (6)	0.75 (0.58–0.88)	25.0
6	Uberlândia	1777	15.34 (8)	0.53 (0.44–0.63)	17.7
7	Uberaba	2578	11.75 (8)	0.73 (0.57–0.86)	24.3
Porto Alegre strain[a]	São Paulo	1057	10.41 (6)	0.03 (0.02–0.03)	–

n, number of *R. microplus* larvae used for LC_{50} calculation; χ^2 (df), Chi square (degrees of freedom); RR (resistance ratio), LC_{50} from field population/LC_{50} from 'Porto Alegre' reference strain.
[a] Susceptible 'Porto Alegre' reference strain.

and Costa (1980), a RR greater than 6 to chlorpyriphos is enough to impair the use of this acaricide in the field.

The RR found at this study to both acaricides was higher than those reported by Mendes et al. (2007) and similar to those observed by Mendes et al. (2011). This may be due to differences between the products used at cattle ranches of each survey in last years. At cattle ranches visited by Mendes et al. (2007) amitraz was the main product used in the preceding five years, while at cattle ranches surveyed by Mendes et al. (2011) products used in the preceding three years were mainly mixtures of pyrethroids and organophosphates or pyrethroids alone, similar to what was being used at cattle ranches of the present study (Domingues, 2011).

One population (Table 2, cattle ranch number 5) had a RR almost two fold higher than others. This population was collected in a cattle ranch where acaricide treatments had been performed, for more than one year, with an organophosphate compound exclusively. At this ranch more than 20 acaricides treatments annually had been applied (Domingues, 2011) which may have contributed to the development of resistance to chlorpyriphos.

Regarding the population susceptible to chlorpyriphos (Table 2) it was collected in a cattle ranch where no organophosphates had been used in the preceding years. Acaricides used at this ranch were composed of pyrethroids, macrocyclic lactones and insect growth regulators (Domingues, 2011).

All larvae surveyed by the allele specific PCR described by Guerrero et al. (2001) showed a homozygous susceptible genotype to T2134A substitution (Supplementary Fig. 1), therefore this mutation was not detected in any sample.

In a previous study carried out by Mendes et al. (2010) in the state of São Paulo, Brazil, 14 cattle tick populations from different ranches had been surveyed with a nested PCR to detect the T2134A mutation and the majority of them was homozygous susceptible, while less than 25% were heterozygous or homozygous resistant. No correlation was found between the presence of the mutation and the RR values (Mendes et al., 2010). Andreotti et al. (2011) also did not find the T2134A mutation in three pyrethroid resistant populations of *R. microplus* form Mato Grosso do Sul, Brazil.

Chen et al. (2009) demonstrated that apparently different mechanisms of resistance had developed independently in Mexican and Australian strains since in their study Mexican populations had the T2134A mutation, but it was not found in any of surveyed Australian larvae.In contrast, the C190A mutation was detected in larvae from all field populations at high frequencies, ranging from 82% to 100% (Table 3). The frequency of the C190A mutation has a close correlation ($R^2 = 0.82$) with the LC50 values for

Table 2
Susceptibility of *R. microplus* to chlorpyriphos in the 'Triângulo Mineiro' and 'Alto Paranaíba' regions within the state of Minas Gerais, Southeast Brazil, April 2010.

Cattle ranches	City	Chlorpyriphos			
		n	χ^2 (df)	LC_{50} (CI 95%)	RR
1	Uberlândia	1563	6.41 (5)	0.04 (0.03–0.05)	4.4
2	Uberlândia	1088	11.39 (6)	0.04 (0.03–0.05)	4.4
3	Uberlândia	2338	10.65 (10)	0.05 (0.04–0.05)	5.5
4	Uberlândia	1924	9.03 (7)	0.06 (0.05–0.07)	6.7
5	Uberlândia	1126	9.51 (4)	0.14 (0.09–0.32)	15.6
6	Uberlândia	1224	9.14 (4)	0.02 (0.01–0.03)[b]	2.2
7	Uberaba	901	7.08 (3)	0.05 (0.04–0.09)	5.5
Porto Alegre strain[a]	São Paulo	1278	13.25 (8)	0.009 (0.008–0.01)	–

n, number of *R. microplus* larvae used for LC_{50} calculation; χ^2 (df), Chi square (degrees of freedom); RR (resistance ratio), LC_{50} from field population/LC_{50} from 'Porto Alegre' reference strain.
[a] Susceptible 'Porto Alegre' reference strain.
[b] CI overlap CI of Porto Alegre susceptible strain: susceptible population.

Table 3
Distribution of observed genotypes and allele frequencies for the C190A mutation in sodium channel alleles in different populations of *R. microplus*.

Cattle ranches	City	No. of homozygous Susceptible (% CC)	No. of heterozygous (% CA)	N° of homozygous Resistant (% AA)	% *C*	% *A*	Total of larvae surveyed
1	Uberlândia	3 (8%)	0 (0%)	35 (92%)	8%	92%	38
2	Uberlândia	0 (0%)	0 (0%)	32 (100%)	0%	100%	32
3	Uberlândia	2 (6%)	8 (24%)	24 (71%)	18%	82%	34
4	Uberlândia	0 (0%)	0 (0%)	34 (100%)	0%	100%	34
5	Uberlândia	2 (5%)	3 (8%)	33 (87%)	9%	91%	38
6	Uberlândia	0 (0%)	5 (14%)	32 (86%)	7%	93%	37
7	Uberaba	2 (6%)	1 (3%)	31 (91%)	7%	93%	34
8	Ituiutaba	0 (0%)	1 (3%)	31 (97%)	2%	98%	32
9	Uberaba	0 (0%)	1 (3%)	33 (97%)	1%	99%	34
10	Campo florido	1 (3%)	3 (10%)	26 (87%)	8%	92%	30
Porto Alegre	São Paulo	1 (50%)	14 (37%)	5 (13%)	68%	32%	38

Alleles with C at position 190 are linked to the susceptible phenotype; Alleles with A at position 190 are linked to the resistant phenotype; hence CC, putative susceptible homozygote; CA, heterozygote; AA, putative resistant homozygote.

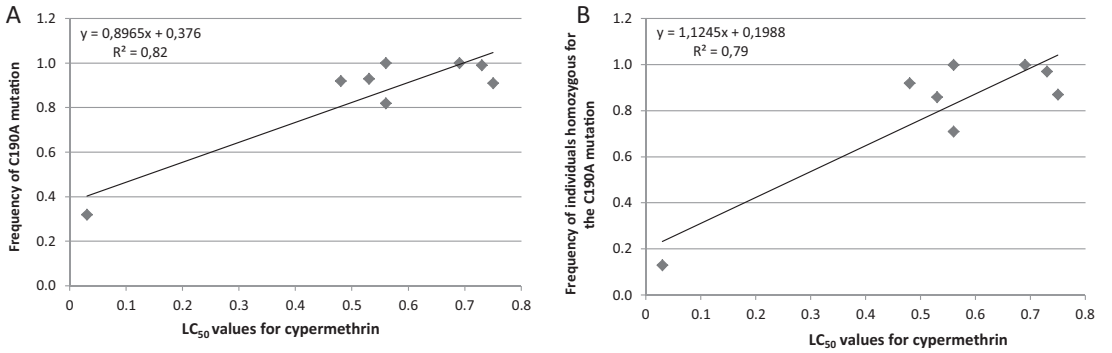

Fig. 1. Correlation between the frequency of the C190A substitution (A) or homozygous C190A genotypes (B) and the LC_{50} values of cypermethrin obtained in the LPT.

cypermethrin (Fig. 1A). In addition, this correlation is maintained at similar levels ($R^2 = 0.79$) when only the frequency of individuals homozygous for the mutation C190A is plotted against the LC_{50} values for cypermethrin (Fig. 1B), corroborating the observation that this is a recessive trait (Morgan et al., 2009).

Despite of the close correlation observed between C190A mutation frequencies and phenotypic resistance to cypermethrin, additional molecular mechanisms, such as other sodium channel mutations or metabolic detoxification, might contribute synergistically to resistance and cannot be ruled out. However, even though the mutation described by Jonsson et al. (2010) was not evaluated here it seems unlikely that it might play a major role in the resistant phenotypes observed, since according to the authors this substitution is related to resistance to flumethrin only, a drug that is not used in Brazil since 1990 decade.

Guerrero et al. (2012) stated that the T2134A mutation is localized to North America, whereas the C190A is widespread to Brazil, Argentina, South Africa and Australia. The results of the present study agree with Guerrero et al. (2012) and contribute to understand the mechanisms involved in pyrethroid resistance in Brazilian cattle tick field populations since more populations were surveyed.

4. Conclusion

This was the first attempt to identify the mechanism of resistance, target site insensibility, in *R. microplus* populations from Minas Gerais state, Brazil. Almost all populations investigated by LPT were shown to be resistant to cypermethrin and chlorpyriphos. The T2134A mutation was not found in any of the 10 samples surveyed, but the C190A was detected in all field populations. This substitution seems to be the main cause of the phenotypic resistance to pyrethroids observed in the bioassays.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2012.04.031

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