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Resistance to deltamethrin, fipronil and ivermectin in the brown dog tick, *Rhipicephalus sanguineus* sensu stricto, Latreille (Acari: Ixodidae)

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ABSTRACT

Rhipicephalus sanguineus sensu stricto (s.s.), the temperate lineage of the brown dog tick, is the most common tick found on dogs from urban areas in Rio Grande do Sul (RS) state, southern Brazil. Chemical treatments against ticks are important to control this pest, but can lead to selection for acaricide resistance. Unfortunately, little is known about acaricide resistance in this tick species in Brazil, although such information is very important to companion animal clinical practice. The objective of this study was to analyze acaricide susceptibility of *R. sanguineus* s.s. from the metropolitan area of Porto Alegre, RS. Engorged females ticks were collected in ten different locations, from naturally infested dogs or the environment (homes, shelters and kennels). The progenies were used in toxicological larval tests with deltamethrin, fipronil and ivermectin. Mortality data was used to determine the median lethal concentrations (LC50) for each tick population and resistance was characterized based on relative susceptibility of the different tick populations against each acaricide. Seven samples were considered resistant to deltamethrin, with resistance ratios (RR) ranging from 2.32 to 5.67. From five tick populations tested with fipronil, three were considered resistant, with RR varying from 2.56 to 13.83. For ivermectin, resistance ratios were lower, ranging from 1.54 to 2.97. The results reveal a notable variance of susceptibility to deltamethrin, fipronil and ivermectin in the *R. sanguineus* s.s. populations studied. This study documents for the first time the existence of acaricide-resistant populations of *R. sanguineus* s.s. in Brazil.

1. Introduction

Rhipicephalus sanguineus sensu lato (s.l.) and *Rhipicephalus sanguineus* sensu stricto (s.s.), known by the common name "brown dog tick", are the most widespread species of ticks in the world (Nava et al., 2018). They are three-host ticks that feed preferably on dogs and occasionally on other hosts such as cats, rodents, birds and humans (Dantas-Torres, 2008). Also, they are responsible for the transmission of pathogens, including *Babesia canis* and *Ehrlichia canis*, causative agents of canine babesiosis and canine monocytic ehrlichiosis. These ticks were also incriminated in the transmission of *Rickettsia rickettsii* (Demma et al., 2005) and *Rickettsia conorii*, agents of human spotted fever (Matsumoto et al., 2005).

Parasitism by *R. sanguineus* is very common in dogs from both urban and rural areas (Dantas-Torres, 2010). This tick is well adapted to human dwellings and heavy off-host infestations can occur in houses (Nicholson et al., 2006) and kennels (Miller et al., 2001); and the tick is frequently found infesting stray dogs in urban areas (Ribeiro et al., 1997). In order to control this tick, veterinarians recommend pet owners to regularly administer topical and systemic acaricides to kill the parasitic life stages (*i.e.* larvae, nymphs and adults). However, environmental application of pesticides is common when high burdens of ticks occur inside households. The use of a specific product rarely, if ever, relies on experimental evidence of susceptibility to the chemicals used. Most of the time, the choice is based on the pet-owner's impression of the efficacy of a given product.

There is no published information about the frequency of acaricide application by pet-owners and/or veterinarians against brown dog tick infestations in Brazil, however, the marketing of these products to control parasites of dogs and cats reached USD 65 million in 2017 (SINDAN, 2018), illustrating the extensive use of these products. Historically, the use of acaricides has led to the selection of resistance in several species of ticks of veterinary importance. The tropical cattle tick, *Rhipicephalus microplus* is, by far, the species of tick with the largest number of reports of acaricide resistance, with extreme cases of ticks resistant to multiple acaricides described in Brazil (Reck et al., 2014) and Mexico (Rodríguez-Vivas et al., 2014). Other rhipicephaline ticks, such as *Rhipicephalus appendiculatus* (Vudriko et al., 2016) and

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Rhipicephalus bursa (Ziapour et al., 2016; Enayati et al., 2009) also have shown resistance against the major classes of acaricides. The first report of acaricide resistance in *Rhipicephalus sanguineus* s.l. was from Panama where ticks of the Corozal strain were found resistant to permethrin, DDT, coumaphos and amitraz (Miller et al., 2001). Later, Borges et al. (2007) described resistance in *R. sanguineus* s.l. from the state of Goiás, west-central Brazil, against cypermethrin, deltamethrin, coumaphos and amitraz. In the southern United States, resistance was reported for the first time for fipronil and permethrin in *R. sanguineus* s.l. from Florida and Texas (Eiden et al., 2015, 2016; Eiden et al., 2017). In the state of Yucatan, southeast Mexico, *R. sanguineus* s.l. was found to be resistant to amitraz, cypermethrin and ivermectin (Rodriguez-Vivas et al., 2017a,b). All these cases of resistance probable occurred in the tropical lineage, *R. sanguineus* s.l. given their geographical distribution. There is only one report of *R. sanguineus* s.s. resistance to acaricides, describing deltamethrin-resistant populations in Spain (Estrada-Peña, 2005). To the best of our knowledge, acaricide resistance has not been reported for the temperate lineage, *R. sanguineus* s.s., in the Americas. Here we provide the first report of acaricide resistance in this species of brown dog tick, collected from naturally infested dogs and their dwellings, in the metropolitan area of Porto Alegre, Rio Grande do Sul, Southern Brazil.

2. Material and methods

2.1. Study area

The study was carried out from May 2015 to June 2016 in three municipalities (Porto Alegre, Viamão and Eldorado do Sul) in the state of Rio Grande do Sul, Southern Brazil. The climate of the area is subtropical humid, with a mean annual temperature of 19.5 ± 3.9 oC, an annual rainfall of 1320 ± 110 mm and a mean relative humidity (RH) of 76% (INMET, 2018). The study area is located within the geographical range of the temperate species of the brown dog tick, *Rhipicephalus sanguineus* s.s. (Nava et al., 2018).

2.2. Tick collection

Ticks (engorged females) were obtained directly from infested dogs and/or their dwellings in 10 different locations. Six populations in Porto Alegre (Centro, Tristeza, Ponta Grossa, Vila Nova, Ipanema and Restinga); three populations in Eldorado do Sul (Sans Souci-BD, Sans Souci-IPV and Eldorado) and one population in Viamão (Viamão). When possible, information about acaricide usage for tick control was obtained from owners/responsible persons for the dogs. Information about the origin of ticks and exposure to acaricides is presented in the Table 1. After collection, ticks were placed inside plastic containers with perforated lids to allow air circulation and transported to the Laboratory of Parasitology at the Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF) located in Eldorado do Sul, Brazil. Upon arrival, ticks were washed with distilled water and identified as *R. sanguineus* s.l. according to morphologic characteristics described by Barros-Battesti et al. (2006). Engorged females were placed in plastic petri dishes and incubated at 26 ± 1 °C and 85% RH to allow egg-laying and hatching. Live larvae, 14 to 28 d of age, were used in the susceptibility bioassays.

Table 1
Origin of ticks, collection sources and history of acaricide exposure.

Municipality	Sample ID	Source	Acaricide exposure
Eldorado do Sul	Sans Souci-IPV	Stray dog	Unknown
	Sans Souci-BD	Stray dog	Unknown
	Eldorado	Dog shelter	Ivermectin; doramectin
Porto Alegre	Centro	Kennel	Fipronil; amitraz
	Vila Nova	Residence	Ivermectin
	Tristeza	Residence	Amitraz; cypermethrin; deltamethrin
	Ponta Grossa	Residence	Unknown
	Restinga	Stray dog	Unknown
	Ipanema	Residence	Ivermectin
Viamão	Viamão	Residence	Fipronil; ivermectin

2.3. Bioassays

2.3.1. Larval packet test

A modified larval packet test (Stone and Haydock, 1962) was used to determine the susceptibility to deltamethrin and fipronil. Initially, technical grade deltamethrin (Sigma Aldrich Co., St. Louis, MO, USA) and Fipronil (BASF, Paulínia, Brazil) were dissolved at 1% (v/v) in a mixture of trichloroethylene (Synth, Diadema, Brazil) and olive oil (2:1) (TchE-OO) to form stock solutions. This stock solution was diluted in TchE-OO in order to obtain a series of concentrations of the active ingredients resulting in a gradient of mortalities between 5 and 95%. Concentrations for deltamethrin were (in ppm): 800, 400, 200, 100, 80, 60, 40, 20 and 10; for fipronil, the following concentrations were used (in ppm): 100, 50, 25, 12.5, 6.25, 3.125 and 1.5265. Aliquots of 0.7 mL of each solution were used to treat pieces of filter paper (No.1, Whatman Inc., Maldstone, UK; 75 mm x 85 mm). Filter papers were allowed to dry for 2 h in a fume hood. After drying, approximately 100 larvae were placed into each filter paper packet, the papers were sealed with metal clips and incubated at 26 ± 1 °C and 85% RH for 24 h. Three replicates and a control (filter paper with trichloroethylene and olive oil) of each concentration of each acaricide were used. After 24 h the packets were opened, and live and dead larvae were counted.

2.3.2. Larval immersion test

The susceptibility to ivermectin was determined with a modified larval immersion test (Klafke et al., 2012). Technical grade ivermectin (22,23 dyhidroavermectin B1, Sigma Aldrich Co., St. Louis, MO, USA) was dissolved at 1% in acetone PA ACS (Merck) to prepare a stock solution. The stock was diluted 1:100 to prepare an initial solution of ivermectin 100 ppm in a diluent containing 1% acetone and 0.02% Triton X-100 (Sigma-Aldrich) in distilled water. The initial solution was used to prepare 10 mL of the following concentrations using the same diluent (in ppm): 36, 24, 15, 7, 5, 3, and 2. One milliliter of each solution was transferred to 1.5-mL microcentrifuge tubes. Approximately 100 larvae were transferred to each tube with a paintbrush, the tube was closed and shaken gently for 10 min. After 10 min, larvae were recovered with a clean paintbrush, allowed to dry in a piece of filter paper and then transferred to a filter paper (85 mm × 75 mm) folded in the half. Packets were sealed with metal clips and incubated at 26 ± 1 °C and 85% RH for 24 h. Three replicates of each concentration and a control (larvae immersed in diluent only) were used. After 24 h the packets were opened, and live and dead larvae were counted.

2.4. Data analysis

Mortality data was submitted to a Probit analysis following Finney (1980), using the software Polo Plus v1.0 (LeOra Software, Berkeley, CA) in order to calculate the median lethal concentration (LC50) with its respective confidence limits of 95% (CL95%) and slope of the regression lines for each tick population and acaricide. The difference of response to treatment in each tick population was considered significant when their CL95% did not overlap. In the absence of a susceptible reference strain, the resistance ratios (RR) were calculated using the formula proposed by Robertson et al. (2006) based on the tick population with the lowest LC50 for each acaricide (Ziapour et al., 2016). The populations were categorized as susceptible when the RR was lower than 1.5, with incipient resistance when the RR was between 1.5 and 2 and resistant when the RR was higher than 2 (Castro-Janer et al., 2010).

Table 2
Results of the larval packet tests with deltamethrin against *R. sanguineus* s.s. from Porto Alegre metropolitan area, Brazil.

Isolate	N	X2 (df)	slope (SE)	LC50 (CL95%) (ppm)	RR
Restinga	2697	115.88 (28)	1.93 (0.135)	173.338 (150.694 – 199.359) a	5.67
Eldorado	2821	67.070 (25)	2.445 (0.141)	138.551 (117.924 – 159.899) ab	4.53
Centro	964	38.043 (16)	1.609 (0.11)	113.985 (83.614 – 149.307) ab	3.73
Ponta Grossa	410	16.343 (7)	1.688 (0.165)	91.664 (55.510 – 136.885) bc	2.99
Ipanema	1458	39.086 (16)	2.092 (0.165)	79.483 (61.239 – 97.205) c	2.61
Tristeza	3350	278.59 (33)	1.787 (0.066)	72.195 (56.874 – 89.541) c	2.36
Vila Nova	848	38.746 (7)	1.308 (0.109)	70.926 (62.496 – 82.081) c	2.32
Sans Souci-BD	1244	36.733 (16)	1.77 (0.118)	36.179 (26.253 – 46.486) d	1.18
Sans Souci-IPV	806	40.482 (8)	1.973 (0.135)	30.562 (19.206 – 43.404) d	–

N = number of larvae, X2: chi-square; df: degrees of freedom; SE: standard error; LC50: median lethal concentration; CL95%: confidence limits of 95%; a, b, c: equal letters correspond to equal LC50 values according CL95% overlap.

3. Results

The results of larval packet tests with deltamethrin for nine different populations of *R. sanguineus* s.s. collected from the study area are shown in Table 2. The LC50 of the most susceptible isolate (Sans Souci-IPV) was 30.562 ppm. The RR of the tested populations ranged from to 5.67 showing that among the nine populations tested, only one, Sans Souci-BD, was considered susceptible. The resistant isolates showed low level of resistance, with RR varying from 2.32 (Vila Nova strain) to 5.67 (Restinga strain). The only population with confirmed exposure to synthetic pyrethroids was the Tristeza strain, and its bioassay resulted in a RR of 2.36.

A more diverse phenotypic response was observed in the assays with fipronil (Table 3). Population Eldorado was the most susceptible, with a LC50 of 1.994 ppm and was used as a reference for the fipronil assays. The LC50 of the other four populations ranged from 2.305 to 13.827 ppm. Only one population was considered susceptible (Tristeza strain) and presented no history of previous exposure to fipronil. The only population with confirmed exposure to fipronil (Centro strain) was considered resistant (RR = 2.56). The other two populations were collected from dogs with no information regarding fipronil usage (Ponta Grossa, RR = 13.83; Sans Souci-IPV, RR = 6.85).

Regarding ivermectin (Table 4), the most susceptible population was Sans Souci-IPV, collected from a stray dog, with a LC50 value of 8.706 ppm. Population Sans Souci-BD, also obtained from engorged females collected from a stray dog presented a slightly higher LC50, with a RR of 1.54, and is categorized as with “incipient resistance”. Ipanema, Eldorado and Viamão strains came from engorged females collected in locations where the responsible parties declared the off-label use of ivermectin to control ticks. Ipanema ticks showed a higher LC50 than Sans Souci-IPV, however the RR was as low (RR = 1.6), whereas Viamão ticks showed the higher LC50 value for ivermectin among the populations tested with a RR of 2.97. Eldorado ticks were collected in two different occasions; ticks from the first collection (Eldorado 1) were obtained from the walls of the dog shelter and presented a LC50 1.77 times higher than the susceptible reference. However, the ticks from the second collection (Eldorado 2) were obtained from a dog that had been treated with doramectin (a macrocyclic lactone) two weeks before the sampling. The LC50 value for the larvae of the second collection (21.672 ppm) was significantly higher than the LC50 of the first collection (14.524 ppm), showing the effect of treatment on the selection of ivermectin resistant ticks.

4. Discussion

In the present study, toxicological bioassays with acaricides were carried out with larvae of *R. sanguineus* s.s. ticks collected in Rio Grande do Sul state, Brazil. Our results show the presence of deltamethrin, fipronil and ivermectin resistant populations in the metropolitan area of Porto Alegre and, to the best of our knowledge this is the first documentation of *R. sanguineus* s.s. resistance to deltamethrin, fipronil and ivermectin in Brazil.

The first finding of pyrethroid resistance in brown dog ticks was reported in 2001 in Panama against permethrin (Miller et al., 2001). Later, cypermethrin-resistant ticks were found in Goiás state, Central Brazil (Borges et al., 2007). Eiden et al. (2015) reported high levels of permethrin resistance in *R. sanguineus* s.l. populations in Texas and Florida (U.S.A.) and recently Rodriguez-Vivas et al. (2017a) described the first report of cypermethrin resistance in Mexico. As in the tropical cattle tick, *R. microplus*, resistance to pyrethroids is likely to be related to mutations in the para-sodium channel encoding gene (Klafke et al., 2017), however, metabolic detoxification also plays a role in resistance (Eiden et al., 2017). Deltamethrin can be delivered directly to dogs by topical formulations (shampoo, spray, pour-on, impregnated collars) or it can be used in environment (households, sheds, kennels). Synthetic pyrethroids have been extensively used to control ticks and other veterinary and urban pests since the 1990’s and it is likely that brown dog tick populations were directly and indirectly exposed to these compounds for several years, resulting in selection for resistance. Contrary to what was observed in Mexico (Rodriguez-Vivas et al., 2017a) and in the United States (Eiden et al., 2015), the pyrethroid resistance level found among the populations collected in the study area was low (RR ranging from 2.32 to 5.67; Table 2). The only population with confirmed history of exposure to pyrethroids was Tristeza, showing a RR of 2.36. The only strains considered susceptible to deltamethrin (Sans Souci-IPV and Sans Souci-BD) were collected from stray dogs found in the same neighborhood and for obvious reasons it is impossible to confirm previous exposure to any pesticide. As the LC50 of both isolates

Table 3
Results of the larval packet tests with fipronil against *R. sanguineus* s.s. from Porto Alegre metropolitan area, Brazil.

Isolate	N	X2 (df)	slope (SE)	LC50 (CL95%) (ppm)	RR
Ponta Grossa	4555	9.307 (20)	4.477 (0.573)	27.573 (20.875 – 33.855) a	13.83
Sans Souci-IPV	1464	153.12 (21)	1.524 (0.07)	13.655 (9.802 – 19.337) b	6.85
Centro	3661	12.468 (22)	5.414 (0.607)	5.109 (4.070 – 6.405) c	2.56
Tristeza	3984	2.969 (20)	4.725 (0.376)	2.305 (2.121 – 2.522) d	1.16
Eldorado	2911	57.045 (22)	3.358 (0.138)	1.994 (1.819 – 2.181) e	–

N = number of larvae, X2: chi-square; df: degrees of freedom; SE: standard error; LC50: median lethal concentration; CL95%: confidence limits of 95%; a, b, c, d, e: equal letters correspond to equal LC50 values according CL95% overlap.

Table 4
Results of the larval immersion tests with ivermectin against *R. sanguineus* s.s. from Porto Alegre metropolitan area, Brazil.

Isolate	N	X2 (df)	slope (SE)	LC50 (CL95%) (ppm)	RR
Viamão	2261	44.426 (22)	2.878 (0.3)	25.846 (21.25 – 36.288) a	2.97
Eldorado 2*	1437	43.245 (14)	3.830 (0.448)	21.672 (18.762 – 26.910) a	2.49
Eldorado 1**	2103	60.81 (25)	4.752 (0.314)	14.524 (13.331 – 15.664) b	1.67
Ipanema	1114	36.052 (16)	2.721 (0.204)	13.887 (11.942 – 16.07) b	1.59
Sans Souci-BD	1149	116.23 (16)	4.431 (0.395)	13.381 (9.852 – 16.305) b	1.54
Sans Souci-IPV	1990	95.539 (22)	4.070 (0.147)	8.706 (7.905 – 9.574) c	–

N = number of larvae, X2: chi-square; df: degrees of freedom; SE: standard error; LC50: median lethal concentration; CL95%: confidence limits of 95%; a, b, c: equal letters correspond to equal LC50 values according CL95% overlap.

* Larvae from females collected on doramectin treated dog.

** Larvae from females collected on the walls of dog shelter.

were equivalent we can infer that they correspond to the same original population occurring in that area. Among the more resistant strains, population Eldorado (RR = 4.33) was collected in a dog shelter, which receives stray dogs from different locations, so it is possible that an introduced resistant population contributed with some level of deltamethrin resistance. Although there was no confirmation of previous pyrethroids exposure in the other resistant isolates (Vila Nova, Ipanema, Ponta Grossa and Centro) we can suppose the introgression of resistant genotypes in those populations, contributing with the resistance phenotype.

Fipronil has been one of the most used compounds to control external parasites of companion animals, especially fleas and ticks. The first report of fipronil resistance in *R. sanguineus* s.l. was registered in the United States in populations of ticks that were collected from dogs treated with spot-on formulation of this acaricide (Eiden et al., 2015). Nevertheless, the authors considered the RRs too low (ranging from 1.72- to 3.52-fold) to be considered resistant and categorized those populations as tolerant to fipronil. These resistance levels can be considered moderate comparing to the population Ponta Grossa, that showed 13-fold resistance to fipronil. This population was collected from dogs frequently submitted to treatments with spot-on formulation of fipronil which can explain the selection for resistance and the higher resistance ratio. To the best of our knowledge, this is the first report of fipronil resistance in *R. sanguineus* s.s. in Brazil.

Ivermectin is one of the most used drugs to control parasites of cattle and has been used off-label to control dogs ectoparasites as *Sarcoptes scabiei*, *Otodectes cynotis* and *Demodex canis* (Campbell, 2016). The extensive use to control internal and external parasites of dogs contributed to the development of ivermectin resistance in Mexico (Rodriguez-Vivas et al., 2017b). Five populations were tested for ivermectin (Eldorado, Ipanema, Viamão, Sans Souci-BD, Sans Souci-IPV) with resistance ratios ranging from 1.54 (Sans Souci-BD, incipient resistance) to 2.97 (Viamão, resistant). Rodriguez-Vivas et al. (2017b) found brown dog tick populations with higher resistance ratios in Yucatan, Mexico (RR up to 30.5). The differences among the resistance levels among populations might be related to the frequency of exposure to macrocyclic lactones, as observed in the tropical cattle tick, *R. microplus* (Klafke et al., 2010; Fernández-Salas et al., 2012). Ivermectin resistance was evident in the population Eldorado where ticks collected after dogs were treated with doramectin, a macrocyclic lactone compound, showed a LC50 (21.672 ppm; 95% CL = 18.762 to 26.910) significantly higher than the ones collected in the first visit to the shelter, before animals received doramectin treatments (LC50 14.524 ppm; 95% CL = 13.331 to 15.664). Resistance to ivermectin in *R. microplus* has been associated with the increase of the detoxification activity of ABC transporters (Pohl et al., 2010; Le Gall et al., 2018), and it has been suggested that ABC transporters may also play a role in ivermectin detoxification in *R. sanguineus* s.l. (Cafarchia et al., 2015). The full comprehension of the resistance mechanisms of ivermectin resistance in *R. sanguineus* s.l. remains to be determined and further studies are encouraged after the reports of ivermectin resistance in this tick species.

The set of results showed here demonstrate the need for reevaluation of tick control strategies for dogs. First, it is necessary to draw attention for the acaricide resistance issue. Secondly, there is an urgent need for more studies evaluating the acaricide resistance of dog ticks from other localities, as well as, more laboratories able to offer tests to diagnose acaricide resistance. Finally, it is necessary to mobilize practitioners and pharmaceutical industry to avoid the spread of tick resistant populations and to assure a better and longer use of chemical acaricides currently available. The example of long-term bad use of control strategies and widely spread acaricide resistance in cattle tick *R. microplus* should be always kept in mind.

The phenomenon of acaricide resistance in *R. sanguineus* s.l. is getting more attention, as their role in transmission of disease agents to humans is increasingly investigated. Efficient control of this tick is necessary to avoid nuisance and transmission of pathogens to companion animals and humans and the correct application of acaricides depends on rational use of the products available. The adaptation and development of techniques to detect acaricide resistance is a fundamental component of the rational control of ticks and should be pursued for the different species of ticks of veterinary and medical importance.

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Conflict of interest

None.

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