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Prevalence of and potential risk factors for multiple resistance to acaricides in *Rhipicephalus (Boophilus) microplus* ticks: A survey in the state of Rio Grande Do Sul, Brazil

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Abstract

The cattle tick *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Ixodida, Ixodidae) is responsible for significant economic losses in bovine production in tropical and subtropical regions worldwide. Control of this tick predominantly involves the use of chemical acaricides; however, their indiscriminate use has led to the selection of resistant populations. A survey on tick populations was conducted in the state of Rio Grande do Sul, in Brazil, to assess the prevalence of multiple resistance to acaricides in cattle farms. Additionally, questionnaires were administered to identify potential risk factors associated with multiple resistance to acaricides. In total, 176 farms with a bovine population of ≥40 cattle were randomly assigned for tick sampling. The resistance to six acaricidal compounds was investigated by bioassays. A larval packet test was performed for amitraz, chlorpyrifos, cypermethrin, fipronil and ivermectin. Fluazuron was screened using an adult immersion test. Multiple resistance to acaricides (i.e., resistance to three or more compounds) was detected in 173 samples, representing 98% of the total samples. Among these samples, 125 (71%) showed resistance to all six compounds tested. Additionally, we classified the resistance intensity into four levels (I to IV) based on the quartile distribution of the bioassay data. Ten samples (6%) showed high and very high levels (III and IV) of resistance to all six compounds tested. Three variables were significantly associated with multiple resistance to the six acaricides tested: (i) use of injectable acaricides to control ticks, (ii) application of more than five acaricide treatments per year, and (iii) farms with larger herds (≥232 animals). These results regarding widespread resistance and the emergence of multiple resistance to acaricides ticks are alarming and highlight the significant challenge of tick control in southern Brazil.

KEYWORDS
bioassays, cattle ticks, control

INTRODUCTION

The cattle tick, *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) (Ixodida, Ixodidae), is the main ectoparasite adversely impacting the cattle industry in tropical and subtropical areas globally (Estrada-Peña & Salman, 2013; Shakya et al., 2020). In 2014, the potential annual productivity losses attributed to this tick in Brazil were estimated to be USD 3.24 billion (Grisi et al., 2014). Tick control on livestock predominantly relies on chemical acaricides (Jongejan & Uilenberg, 2004); however, their continued and indiscriminate use can lead to the selection of resistant tick populations (Klafke et al., 2017).

Resistance can be described as the process of selecting populations with specific heritable characteristics after exposure to a drug, resulting in reduced susceptibility to its effects (Devaney, 2013; Obaid et al., 2022). Resistance occurs through toxicodynamic changes, which involve modifications at the target site, or toxicokinetic changes, which entail reduced penetration and increased detoxification (de Rouck et al., 2023). Resistance results in ineffective treatment and increases the cost of tick control. Reports from Brazil have described tick populations resistant to six classes of acaricides (Klafke et al., 2017; Reck et al., 2014), with the exception of isoxazolines (introduced to the market in 2022). Worldwide, the most alarming outcome is the emergence of populations showing multiple resistance to acaricides. This phenomenon has been also observed in Colombia, Mexico, India and Ecuador (Benavides et al., 2000; Fernández-Salas et al., 2012; Klafke et al., 2017; Pérez-Otáñez et al., 2024; Sagar et al., 2020).

For diagnosis of resistance in ticks, the larval packet test (LPT) is the most frequently utilised bioassay reported in the literature (Abbas et al., 2014; Food and Agriculture Organization [FAO], 2004). Other tests, such as the larval immersion test and the adult immersion test (AIT), can also provide rapid and valuable guidance in detecting the emergence of resistance, helping to anticipate control failure in field situations (Abbas et al., 2014; Chaparro-Gutiérrez et al., 2020). In addition, the use of diagnostic tools is crucial for managing resistant tick populations, mitigating the escalating issue of multiple resistance to acaricides and developing new strategic control programs. Active surveys can offer valuable insights into the prevalence and distribution of resistant tick populations in the field. Furthermore, this information provides valuable guidance for decision-making processes regarding tick control on farms and the development of surveillance and control measures by the state veterinary service, helping to mitigate the emergence and spread of resistant ticks.

We conducted a random survey on tick populations from Rio Grande do Sul state, southern Brazil, with the aim of assessing the prevalence of multiple resistance to acaricides in cattle ranches. Additionally, questionnaires were administered to farmers to identify potential risk factors associated with multiple resistance to acaricides.

MATERIALS AND METHODS

Study design

The current survey was conducted in Rio Grande do Sul in southern Brazil. The climate of the region is classified as subtropical temperate according to the Köppen scheme. The annual mean temperature ranges from 15 to 18°C, with a maximum temperature of 40°C, and the annual rainfall ranges from 1299 to 1800 mm (SPGG, 2021). The state exhibits two distinct types of biomes: Pampa, predominantly found in the southern region and covering 68.8% of the state, and the Atlantic Rainforest, situated in the northern region (IBGE, 2019).

This study was designed as a prevalence survey to elucidate the situation of multiple resistance to acaricides among ticks on cattle ranches in Rio Grande do Sul. The total number of cattle farms in the state was acquired from the state veterinary service. The farms included in this study have at least 40 animals to increase the odds of gathering the minimum number of ticks required for bioassays. The total number of farms with more than 39 cattle was 52,615. The sampling calculation was conducted using a random one-stage strategy, incorporating a correction factor for finite populations (Serdar et al., 2021). The following formula was used: number to be sampled $(n) = \{[Z2 \times P(1-P)]/E2\}/\{1 + [Z2 \times P(1-P)]/E2 \times N\}$, where Z is the score for a 90% confidence level (i.e., 1.65), E is the margin of error (0.05), P is the estimated prevalence of multiple resistance (0.80 according Klafke et al., 2017) and N is the total population size (52,615 farms). Thus, the calculated sample size (n) was 174 farms.

Next, 302 farms were randomly raffled from the total of 52,615 ranches using a web-based calculator (https://www.calculator.net/sample-size-calculator.html). Farmers were informed about the study goals and consented to participate in the study. All 302 farms were visited by state officers, and where possible, approximately 100 engorged female *R.* (*B.*) *microplus* ticks were collected from each farm. Additionally, a questionnaire was administered to farmers. This study was approved by the local committee on animal experimentation (CEUA-SEAPDR 007/2022).

Preparation of ticks

Engorged female ticks were collected from the hosts and subsequently transported to Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Eldorado do Sul, Rio Grande do Sul, Brasil, inside plastic containers within 48 h. The ticks were processed in accordance with the recommendations of the Food and Agriculture Organisation of the United Nations (2004). The ticks were washed with distilled water and dried with paper towels. The ticks were then separated into two groups for the AIT and LPT. Ticks used for the AIT were processed immediately after arrival at the laboratory. Ticks used for the LPT (groups of 30–40 engorged female ticks) were incubated in plastic Petri dishes in an incubator at 27 to 28°C and 80% to 90% relative humidity for a period of 2 weeks. Once laid eggs, within one dish were carefully homogenised, separated in aliquots of 500 mg and incubated in 5-mL serum vials (diameter, 23 mm; height, 47 mm) closed with cotton plugs under the same conditions to allow the larvae to hatch. LPT were performed with 14- to 21-day-old larvae.

Chemicals

Bioassays were performed with the following technical-grade acaricides: chlorpyrifos, cypermethrin, fipronil, fluazuron and ivermectin (Sigma Chemical Corporation, St. Louis, MO, USA). Amitraz was tested with a commercial formulation at 12.5% (Triatox®; MSD Saúde Animal, São Paulo, Brazil).

LPTs

LPTs were performed according to the method described by Stone and Haydock (1962) with modifications described by Klafke et al. (2017). Briefly, acaricides were diluted in a solution consisting of two parts trichloroethylene (Synth, Diadema, São Paulo, Brazil) and one part commercial olive oil. Each piece of filter paper (85 × 75 mm, Whatman No. 1; Whatman Inc., Maidstone, England) was impregnated with 0.7 mL of respective acaricide solution. The control group was impregnated with trichloroethylene–olive oil only. For trichloroethylene evaporation, the packets were left to dry for 30 min inside a fume hood, then wrapped in aluminium foil and stored at 4°C until use. Each compound was impregnated in batches of 60 packets, with 65 batches processed. For the bioassay, three packets of each acaricide were folded in the middle and sealed with metal clips on both sides. Larvae were carefully transferred into the packets using a flat No. 2 paintbrush, and a third metal clip was used to seal the top. Approximately 100 larvae were tested for each packet. The packets were incubated at 27 to 28°C and 80% to 90% relative humidity. Larval mortality was assessed by counting both dead and living individuals after 24 h; for the packets of amitraz, however, the assay was assessed after 48 h, as recommended by FAO (2004).

Tests were performed using discriminating concentrations (DC) of acaricides according to a previous study that demonstrated this to be an effective method for significantly minimising the work needed to identify acaricide resistance (FAO, 2004). To determine the DC for each acaricide, concentration–response tests were conducted using a range of eight different concentrations for each acaricide with a susceptible reference strain (Porto Alegre) (Reck et al., 2014). The mortality data were submitted to a probit analysis with PoloPlus (LeOra Software, 2004) to estimate the lethal concentration for 50% (LC50) and 99% (LC99) of the population sampled for each acaricide along with its 95% confidence interval (95% CI). The DC was established as 2 × LC99 (FAO, 2004). The DCs for cypermethrin and ivermectin were both 1%, for chlorpyriphos was 0.8%, for amitraz was 0.1% and for fipronil was 0.04%. Information regarding the LC50, LC99 and DC of each acaricide for the Porto Alegre reference strain is available in Supplementary Table 1.

AIT

Fluazuron is an acaricide that functions as a growth regulator and, unlike the other compounds tested in this study, cannot be used in resistance tests involving larvae, as it does not exhibit larvicidal activity in vitro. While the recommended bioassay for the other compounds is the LPT, the only suitable bioassay for fluazuron is the AIT. The bioassays were conducted following the methodology reported by Reck et al. (2014) with some modifications. Technical-grade fluazuron was diluted in 2% Triton X-100 (Sigma Chemical Corporation) in dimethyl sulfoxide (Casa da Química, Diadema, São Paulo, Brazil) to obtain a stock solution of fluazuron at 5%. To perform the AIT, the stock solution was diluted 1:100 in demineralised sterile water to provide 10 mL of immersion solution at a final concentration 0.05% of fluazuron, as previously established (Reck et al., 2014). Three homogeneous groups (control and two fluazuron tests), each consisting of 10 engorged females, were assembled based on weight (maximum difference of weight ± 0.01 g). The engorged females were immersed for 5 min, sieved out, dried with paper towels and incubated in plastic Petri dishes in an incubator (27–28°C and 80% relative humidity) for 2 weeks. Once laid, the eggs were homogenised, weighed and incubated in 10-mL glass vials plugged with cotton and returned to the incubator under the same conditions. After 4 weeks, the percentage of egg hatch from each sample was visually estimated using a stereomicroscope, by comparing the proportion of larvae in relation to the proportion of unhatched eggs (Davey et al., 2003, 2005; Drummond et al., 1973; FAO, 2004; George & Davey, 2004). To maintain accuracy, estimates are provided in 5% intervals by the same trained operator, ensuring consistency across all observations.

The in vitro efficacy was assessed using the following equations: IF = (egg mass weight/engorged female weight) × % larval hatch in vitro efficacy (%) = 1 − [(IF control group − IF treated group)/IF control] × 100, where IF is the index of fertility.

Questionnaires

In addition to collecting samples, questionnaires were administered to farm owners or managers to obtain data on the cattle type, farm structure, handling procedures, acaricide usage, occurrence of tick-borne diseases and tick control strategies. The questionnaire was specifically designed to characterise and identify potential risk factors associated with multiple resistance to acaricides. The cattle information encompassed details such as breed (European, Indian or crossbreeds), cattle production type (beef, dairy, and mixed cattle) and feeding system (native pasture, native and cultivated pasture, cultivated pasture, and feedlots). Regarding the structure of the farm and its handling practices, information was gathered about the presence/absence and usage of dipping vat and spray race, cattle purchase and handling practices following arrival to the farm (quarantine and acaricide treatment), and veterinary assistance (presence/absence of veterinary assistance and tick control managed by a veterinarian). Other aspects of the questionnaire covered methods of acaricide application, frequency of treatments per year, acaricides used and pasture control strategies (field mowing, rotational grazing and spelling pasture periods). Acaricide purchase criteria were investigated, including recommendations from veterinarians, recommendations from other farmers, suggestions from the farm supply store attendant, advertisements, cost considerations, laboratory tests and unknown criteria. Previous laboratory tests also were noted. Additionally, information was gathered on the occurrence of ticks and tick-borne diseases in the last 5 years.

Details regarding the GPS location, region, cattle herd and farm size were obtained from the state veterinary service. The interviews were conducted by state veterinary officers. The questionnaires were administered using the Epicollect5 platform (Centre for Genomic

Pathogen Surveillance, Imperial College, London, UK; https://five.epicollect.net). The full questionnaire is available upon request to the corresponding author.

Analysis of data

Following the laboratory analysis, field samples were categorised as resistant if the mortality was <95%. This value was determined to consider a potential margin of error of the test based on values obtained from the mean absolute deviation among the assay replicates.

For data analysis, the results of the resistance tests were classified into four resistance levels. The results of tests using amitraz, chlorpyriphos, cypermethrin, fipronil and ivermectin were categorised based on larval mortality. This categorisation was established through quartile analyses utilising Microsoft Excel command 'QUARTILE.INC'. The quartiles were derived from the entire dataset of larval bioassays for all five compounds mentioned above. The four levels of resistance were Level I (<95% and ≥ 85% mortality), Level II (<85% and ≥ 48%), Level III (<47% and ≥ 18%) and Level IV (<18%). For fluazuron, the samples also were categorised as resistant if the in vitro efficacy was <95%. Consequently, we calculated specific quartiles based on the fluazuron test outcomes. The resistance levels for fluazuron were Level I (<95% and ≥54% in vitro efficacy), Level II (<54% and ≥29%), Level III (<29% and ≥13%) and Level IV (<13%).

The associations between the risk factors (independent variables) and simultaneous multiple resistance to six acaricide compounds (dependent variable) were examined through univariate analysis (chi-square test or Fisher's exact probability test) using VassarStats (http://www.vassarstats.net/odds2x2.html). Odds ratio (OR) analyses also were performed using the VassarStats platform. Variables with a p value of <0.2 in the univariate analysis were further analysed by binomial multivariate logistic regression and OR analysis using the statistical package SPSS version 20 (IBM SPSS Statistics Base v.20; IBM Corp., Armonk, NY, USA). Independent variables with p values of <0.05 were deemed statistically significant when associated with the dependent variable.

The risk factor analysis was performed with consideration of the following independent variables: region (southern region or northern region of the state), cattle type production (beef cattle production; dairy cattle and mixed cattle productions), cattle type operation (calf and stocker cattle; others), cattle breed (European breeds; crossbreeds and Indian breeds), cattle reposition (external sources or internal sources), cattle herd size (≥232 or <232 animals per farm), integrated tick control (use or not of rotational grazing, spelling pasture, and/or crop-livestock system [cattle farming operation integrated with grain agriculture]), use of injectable formulations (yes or no), frequency of acaricide treatments per year (more than five acaricide treatments, or up to five acaricide treatments) and veterinary assistance (yes or no).

The location of each farm studied was plotted using the geographical information system software ArcGIS 10.5 (ESRI, Redlands, CA, USA). A kernel-density interpolation function in the Spatial Analyst extension of ArcGIS software was used to convert point data into continuous surfaces, expressing the intensity per square kilometre of the occurrence of a tick population (in a farm) showing multiple resistance to six acaricides. The kernel-density themes were estimated using a non-fixed Silverman's bandwidth (Silverman, 1986) (range, 4–30 km) for the kernel-density function, allowing the generation of a heat map for identification of geographical hot spots for ticks with multiple resistance to acaricides within the studied population.

RESULTS

Tick samples from 176 farms were included in this study. Regarding the characteristics of the farms sampled, the predominant genetic compositions of the cattle were European (51%, 89/176) and crossbreeds (48%, 85/176); only 1% of the farms had Indian Zebu breeds (2/176). Most of the farms (89%, 156/176) were engaged in beef cattle production. The average number of cattle was 232 animals (range, 40–1671), with a stocking rate of 379 kg/ha in the southern region and 282 kg/ha in the northern region. A significant portion of the farms (61%, 108/176) integrated native and cultivated pasture areas, while 32% (57/176) exclusively maintained native pasture areas. Approximately 51% (90/176) of the farms operated with a full cattle cycle, and 40% (71/176) focused on calf and stocker cattle operations. Among the 302 farms visited, 22 (7%) respondents stated that their farms were currently free of cattle ticks.

In terms of tick control, a substantial majority of the farms (65%, 115/176) implemented integrated strategies, including rotational grazing, spelling pasture and/or a crop-livestock system. Most of the farmers (65%, 114/176) acquired cattle from external sources and applied chemical acaricide treatments (77%, 88/114) to newly purchased or returning animals; 40% (46/114) adopted quarantine measures with the treatments. All farmers utilised acaricides for tick control. The predominant methods of acaricide application were pour-on formulations (86%, 151/176) and injectable formulations (mainly ivermectin and doramectin) (81%, 142/176). Additionally, manual spraying (mainly synthetic pyrethroids) was employed in 33% (58/176) of the farms, while dipping vats (mainly amitraz and mixture of synthetic pyrethroids and organophosphates) were utilised in 28% (50/176). Figure S1 shows the most frequently used compounds among the 176 sampled farms as a word cloud diagram. Almost 51% (89/176) of the farms operated without regular veterinary assistance. Only 36% (64/176) of the respondents declared that tick control was managed by a veterinarian. Regarding the frequency of acaricide application, 57% (100/176) of farmers used more than six acaricide treatments on cattle per year, while approximately 20% of respondents reported that they had used 12 acaricide treatments or more in the past year. The maximum number of acaricide treatments per year was 22. With respect to the occurrence of tick-borne diseases, 68% (121/176) of the farmers reported cases of babesiosis or anaplasmosis, with lethalities reported in 40% of farms (70/176).

Bioassays to determine acaricide resistance were conducted on 176 tick samples (Table 1). Resistance to cypermethrin, ivermectin,

TABLE 1 Relative frequency of samples of *Rhipicephalus (Boophilus) microplus* tick populations susceptible and resistant to acaricides among samples randomly collected in Rio Grande do Sul state, Brazil ($n = 176$). Data was based on larval bioassays with cypermethrin, ivermectin, fipronil, chlorpyrifos, amitraz and adult immersion test with fluazuron.

Acaricide	Susceptible	Resistant
Cypermethrin	0 (0/176)	100% (176/176)
Ivermectin	0.6% (1/176)	99.4% (175/176)
Fipronil	4% (7/176)	96% (169/176)
Chlorpyrifos	7.4% (13/176)	92.6% (163/176)
Amitraz	14.8% (26/176)	85.2% (150/176)
Fluazuron	17% (30/176)	83% (146/176)

fipronil, chlorpyrifos, amitraz and fluazuron was observed in 176 (100%), 175 (99.4%), 169 (96.0%), 163 (92.6%), 150 (85.2%) and 146 (83.0%) samples, respectively. For each compound, the distribution of farms with ticks among the four resistance levels exhibited significant variability, as illustrated in Figure 1. Most of the populations of ticks demonstrated level IV resistance to cypermethrin (56%, 99/176), with none of the samples categorised as susceptible or showing low-level resistance to this synthetic pyrethroid. Level III resistance to ivermectin was more prevalent (39%, 69/176), and only one of the samples was susceptible. For fipronil, the frequencies of level III and IV resistance were 30% (51/176) and 35% (63/176), respectively. Chlorpyrifos resistance was predominantly level I (33%, 58/176). For amitraz, 14% (26/176) of the samples were susceptible, and 43% (77/176) displayed level I resistance. For fluazuron, the predominant resistance levels observed were III and IV, with frequencies of 24% (43/176) and 27% (49/176), respectively.

Table 2 shows the frequency of multiple resistance to three, four, five and six acaricides among the tested samples. Multiple resistance to three compounds simultaneously was identified in three (1.7%) field samples. Additionally, 12 (6.8%) samples exhibited resistance to four compounds, while 33 (18.8%) samples displayed resistance to five compounds. Furthermore, 125 (71.0%) samples showed resistance to all six compounds tested. Multiple resistance (i.e., resistance to three or more compounds) was evident in 173 (98.3%) samples. Figure 2 shows maps of the distribution of the sampled farms, highlighting the distribution of cases of presence of ticks with multiple resistance to acaricides by categories (Figure 2a) and hot spots of multiple resistance to six acaricides (Figure 2b). These hot spots appeared to be more highly concentrated in the southern part of Rio Grande do Sul, within the Pampa biome. Ten (0.06%) samples showed level III and IV resistance to all six compounds.

Regarding the risk factor analysis, the univariate analysis identified five variables statistically associated with increased odds of presence of ticks with resistance to all six acaricide compounds: (i) farms in the southern region of the state (OR = 3.0), (ii) beef cattle farms (OR = 4.5), (iii) the use of injectable acaricides to control ticks (OR = 3.2), (iv) application of more than five acaricide treatments per year (OR = 2.5) and (v) farms with larger herds (232 cattle or more) (OR = 5.2). Table 3 presents all variables analysed by univariate analysis, as well as, their OR, 95%CI and *p* values.

After conducting the univariate analysis, the association of these variables with the presence of ticks with resistance to all six acaricide compounds was further explored through a multivariate analysis (Table 4). The final risk factor model suggested three major variables associated with ticks with multiple resistance to six acaricides. The use of injectable acaricides to control ticks significantly increased the odds by approximately five times. Farmers who applied more than five acaricide treatments per year showed an almost fourfold increase in the odds of presence of ticks with multiple resistance to six acaricides occurring in their properties. Additionally, farms with larger herds (above 231 cattle) had approximately 11 times more odds to have ticks with multiple resistance to six acaricides.

DISCUSSION

Rio Grande do Sul boasts a longstanding tradition in the Brazilian cattle industry. The bovine population in this state was approximately 11.9 million animals in 2022 (IBGE, 2022). Historically, Rio Grande do Sul was recognised as the first state of Brazil to document ticks resistant to organophosphates and synthetic pyrethroids, as well as the first region in the world to register *R. (B.) microplus* resistance to macrocyclic lactones and fluazuron (Arteche, 1972; Laranja et al., 1989; Martins et al., 2006; Martins & Furlong, 2001; Reck et al., 2014). Furthermore, there have been reports of tick populations in Rio Grande do Sul exhibiting resistance to six chemical classes available on the market (Klafke et al., 2017; Reck et al., 2014), with the exception of the newly introduced isoxazolines. Nevertheless, most of the information regarding resistant tick populations is derived from biased diagnostic databases, and it may not accurately reflect the real situation. Random active surveys provide information to guide and enhance actions while minimising sampling bias to describe the characteristics of a population (Heeringa et al., 2017; Saylor et al., 2012). This survey was designed to be representative of farms with more than 39 heads of cattle, which represent 20% of the 287,945 farms in the Rio Grande do Sul state.

The results revealed that none of the populations were susceptible to all the acaricides tested. Additionally, most of the cattle ticks exhibited resistance to six classes of acaricides commonly used for chemical control in Brazil, namely organophosphates (chlorpyrifos), synthetic pyrethroids (cypermethrin), formamidines (amitraz), macrocyclic lactones (ivermectin), phenylpyrazoles (fipronil) and benzoylphenyl ureas (fluazuron). These results indicate a situation even more problematic than that previously observed by Klafke et al. (2017) in the same region. In comparison of the results between the current study and that conducted by Klafke et al. (2017), we observed an increase in the resistance frequency and intensity to cypermethrin, amitraz, chlorpyrifos, ivermectin and fipronil, with the latter three being particularly noteworthy. Additionally, we found an increase in the frequency of multiple resistance to acaricides.

In the present study, acaricide resistance was classified into four different levels. Most populations exhibited high and very high levels

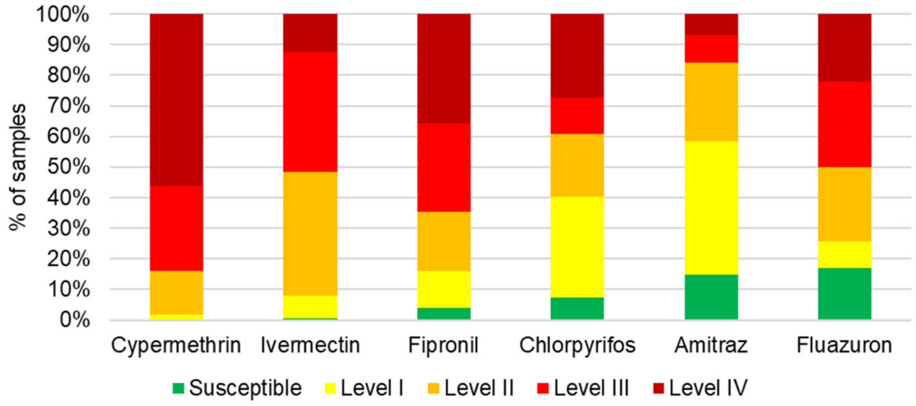

FIGURE 1 Frequency (% of samples) of acaricide susceptible and resistant samples of *Rhipicephalus (Boophilus) microplus* ($n = 176$) from the State of Rio Grande do Sul, Brazil. Four levels of resistance were categorised based on larval mortality for amitraz, chlorpyriphos, cypermethrin, fipronil and ivermectin: (i) Level I: <95% and ≥85% mortality, (ii) Level II: <85% and ≥48%, (iii) Level III: <47% and ≥18%, and (iv) Level IV: <18%. For fluazuron, the levels were (i) Level I: <95% and ≥54% in vitro efficacy, (ii) Level II: <54% and ≥29%, (iii) Level III: <29% and ≥13%, and (iv) and Level IV: <13%.

TABLE 2 Acaricide resistance profiles and multiple resistance to acaricides levels of field samples of *Rhipicephalus (Boophilus) microplus* ($n = 176$) randomly sampled in Rio Grande do Sul state, Brazil.

Acaricide resistance profile	Relative frequency (%) of sample for each profile (*n*)	Multiple resistance to acaricides level	Relative frequency (%) of sample in each level (*n*)	% cumulative frequency of multiple resistance to acaricides (*n*)
-	-	0 (susceptible)	0 (0)	
-	-	1 (single-resistant)	0 (0)	
CYP; IVM	1.7 (03)	2 (double-resistant)	1.7 (03)	
CYP; IVM; FIP	1.7 (03)	3 (resistant to three acaricides)	1.7 (03)	
CYP; IVM; FIP; AMI	2.8 (05)	4 (resistant to four acaricides)	6.8 (12)	8.5 (15)
CYP; IVM; FIP; CHL	1.7 (03)			
CYP; IVM; FIP; FLU	1.7 (03)			
CYP; IVM; CHL; AMI	0.6 (01)			
CYP; IVM; FIP; CHL; AMI	8.5 (15)	5 (resistant to five acaricides)	18.8 (33)	27.3 (48)
CYP; IVM; FIP; CHL; FLU	8 (14)			
CYP; IVM; CHL; AMI; FLU	1.7 (03)			
CYP; FIP; CHL; AMI; FLU	0.6 (01)			
CYP; IVM; FIP; CHL; AMI; FLU	71 (125)	6 (resistant to six acaricides)	71 (125)	98.3 (173)

Abbreviations: AMI, amitraz; CHL, chlorpyrifos; CYP, cypermethrin; FIP, fipronil; FLU, fluazuron; IVM, ivermectin.

of resistance to cypermethrin, ivermectin, fipronil and fluazuron, while they demonstrated predominantly low and moderate levels of resistance to chlorpyrifos and amitraz. This finding is notable considering that chlorpyrifos and amitraz have been used in tick control for more than 50 years. Historically, these compounds were frequently utilised in formulations for immersion dipping vats; however, this method has become infrequently used because of the challenges associated with managing acaricide emulsions as well as concerns about environmental contamination. In the 1980s, there were approximately 4000 vats in use in Rio Grande do Sul (unpublished data from the state

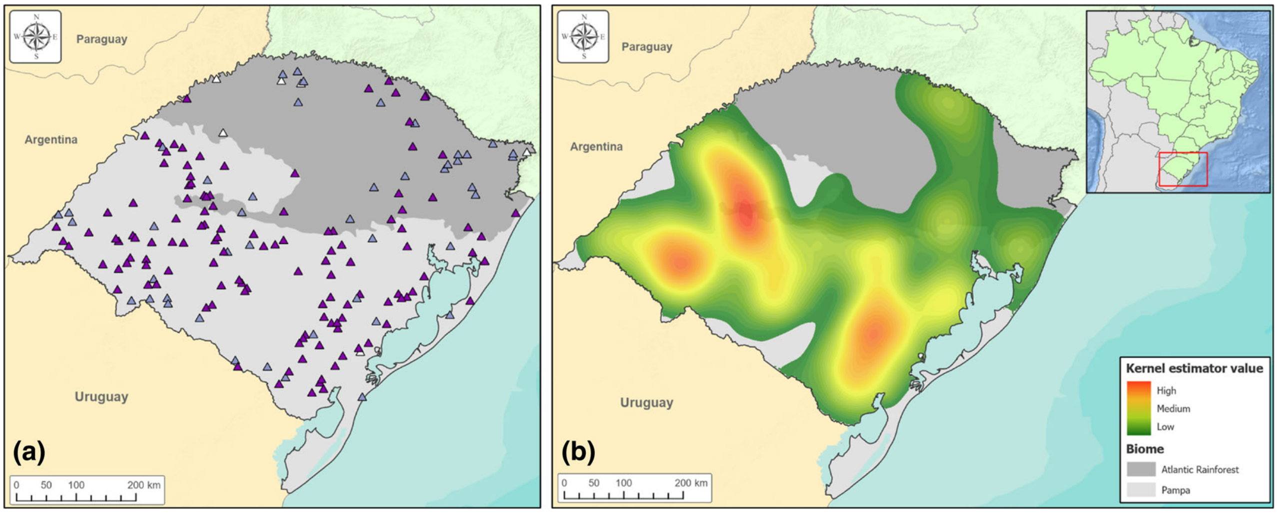

FIGURE 2 Study area map of cases of multiple resistance to acaricides in field samples of *Rhipicephalus (Boophilus) microplus* ($n = 176$) randomly collected in cattle farms from the State of Rio Grande do Sul, Brazil. (a) Distribution of multiple resistance cases. For this spatial analysis, the categories were grouped into three major groups, as following: The white triangles indicate tick populations resistant to two and three acaricide classes, blue triangles indicate those classified as multiple resistance to four and five acaricides, and purple triangles depict multiple resistance to six acaricides populations. The Atlantic Rainforest biome is shown in dark grey, while the Pampa biome is shown in light grey. (b) Kernel density estimate highlighting the hotspots of multiple resistance to six acaricides. Insert indicated Brazil and inside the red rectangle, Rio Grande do Sul state. The colour scales in maps indicate the concentration of farms classified as multiple resistance to six acaricides category in each location (kernel estimator value). The Atlantic Rainforest biome is depicted in dark grey, while the Pampa biome is shown in light grey.

veterinary service). Our survey showed that only 34% of the farms had dipping vats, with less than 25% of them still being used (data not shown).

Klafke et al. (2017) observed a frequency of multiple resistance to acaricides rate of approximately 80% based on a diagnostic database from a reference laboratory in Southern Brazil. To avoid sampling bias, we analysed the presence of multiple resistance using a random sampling approach. Nevertheless, we found that 98.3% of tick samples were resistant to three or more compounds. The observed differences in the frequency of multiple resistance to acaricides between these two studies may be associated with the sampling bias. Samples from the study by Klafke et al. (2017) might have been provided by farmers concerned about tick control and resistance to acaricides. Another possible explanation is that the widespread use of acaricides in the last 7 years has increased the frequency of multiple resistance to acaricides from 80% to 98%. Furthermore, 10 tick populations showed high or very high levels of resistance to all six acaricide compounds. The scenario of high multiple resistance in southern Brazil is alarming (FAO, 2022), and it highlights the significant challenge of tick control using chemicals. A widely recommended strategy for effective tick control is the rotation of acaricides, which involves alternating the use of two or more compounds without evidence of resistance (Rodríguez-Vivas et al., 2018). However, our data on widespread resistance to multiple acaricides suggests that this strategy may not be feasible to be implemented in all farms. Notably, 71% of the tick samples showed some degree of resistance to six out of the seven commercially available chemical classes of acaricides. This underscores the urgent need for new approaches to managing tick resistance on farms. One potential alternative was highlighted by Centenaro et al. (2022), who successfully employed strategic tick control by utilising acaricides with the lowest observed resistance in a tick population exhibiting multiple resistance to all acaricides available at that time.

Quantitative analysis permits the determination of resistance levels and can provide guidance on how to conduct acaricide resistance management. Castro-Janer et al. (2011) proposed three categories for diagnosing resistance (susceptible, incipient resistance and resistant) based on statistical estimation of the lethal concentration for 50% of the population and the resistance ratios values. Because of the large number of samples collected in this survey, we were unable to perform such analysis, which could have improved our understanding of the resistance levels in field tick populations in Rio Grande do Sul. However, when quantitative analysis is unavailable, qualitative and semi-quantitative approaches, as demonstrated by Klafke et al. (2017) and the present study, can help to categorise resistance levels. By utilising quartile analyses based on larval mortality, our method highlighted the degree of resistance, providing valuable guidance for veterinarians and farmers in their decision-making processes concerning tick control.

Regarding risk factors, we identified five variables statistically associated with an increased likelihood of multiple resistance to the six compounds in the univariate analysis and three in the final multivariate model. Concerning the highest frequency of multiple resistance in the southern part of the state, it is noteworthy that this finding is supported by our kernel spatial analysis. The analysis showed hot spots for the presence of ticks with multiple resistance concentrated in the south, within the Pampa biome, which is characterised by high concentration of the bovine population under an elevated stocking rate (Ruviaro et al., 2016). Indeed, these features when

TABLE 3 Univariate analysis of risk factors for occurrence of *Rhipicephalus (Boophilus) microplus* resistant to six acaricide compounds in samples of 176 farms from Rio Grande do Sul state, Brazil.

Independent variable	Category	Resistant	Susceptible	P value	OR (95% CI)[a]
Region[b]	Southern Region	95/121 (79%)	26/121 (21%)	0.001	3.0 (1.5–6.0)
	Northern Region	30/55 (55%)	25/55 (45%)		
Cattle type production[b]	Beef cattle	117/156 (75%)	39/156 (25%)	0.001	4.5 (1.7–11.8)
	Dairy cattle or mixed cattle	8/20 (40%)	12/20 (60%)		
Cattle type operation	Calf and stocker cattle	53/71 (75%)	18/71 (25%)	0.383	-
	Others	72/105 (69%)	33/105 (31%)		
Cattle breed	European breeds	61/89 (69%)	28/89 (31%)	0.462	-
	Crossbreeds and Indian breeds	64/87 (74%)	23/87 (26%)		
Cattle reposition	External sources (purchase)	81/114 (71%)	33/114 (29%)	1	-
	Internal sources	44/62 (70%)	18/62 (30%)		
Cattle herd size[b]	≥232 animals	40/44 (90%)	4/44 (10%)	0.0007	5.2 (1.9–16.4)
	<232 animals	85/132 (64%)	47/132 (36%)		
Integrated tick control	Use of integrated strategies (rotational grazing, spelling pasture and/or crop-livestock system)	80/115 (70%)	35/115 (30%)	0.560	-
	No use of integrated strategies	45/61 (74%)	16/61 (26%)		
Injectable formulations[b]	Use of injectable acaricides	108/142 (76%)	34/142 (24%)	0.003	3.2 (1.5–6. 9)
	No use of injectable acaricides	17/34 (50%)	17/34 (50%)		
Frequency of acaride treatment per year[b]	> five acaricide treatments	79/100 (79%)	21/100 (21%)	0.007	2.5 (1.3–4.8)
	≤ five acaricide treatments	46/76 (61%)	30/76 (39%)		
Veterinarian assistance	No veterinarian assistance	60/89 (67%)	29/89 (33%)	0.286	-
	Presence of veterinarian assistance	65/87 (75%)	22/87 (25%)		

Note: Variables statistically associated with resistance to six compounds showed in bioassay.
Abbreviations: 95%CI, 95% confidence interval; OR, odds ratio.
[a]OR were calculated only for variables with p values <0.05.
[b]p values below 0.05 at univariate analysis.

associated are often linked with high tick loads (Nava et al., 2024). Thus, in higher infestation levels, the demand for acaricide treatment will increase, contributing to the emergence of multiple resistance.

Our results showed a higher prevalence of resistant ticks in beef cattle systems compared to dairy and mixed cattle. This may be attributed to the notable difference in the availability of acaricides for use in these systems, with a wider variety of compounds accessible for tick control in beef cattle (do Nascimento et al., 2021). The greater range of acaricides used in beef cattle is likely to increase the use of different chemical classes, which can be directly related to the selection for multiple resistance.

In the multivariate model, the use of injectable formulations for tick control was significantly associated with an increased likelihood of finding ticks with multiple resistance to acaricides. This may be related to reports from several interviewed farmers (data not shown) who commonly use both injectable and topical acaricides simultaneously to enhance the efficacy of treatments against multiple-resistant ticks. It is reasonable to hypothesise that this practice could facilitate the selection for multiple resistance. To the best of our knowledge, although there is no direct scientific evidence linking this practice to resistance selection, it certainly warrants further investigation.

Regarding the frequency of acaricide treatments, a reduced likelihood of the presence of resistant ticks is expected when fewer treatments are administered (Jonsson et al., 2000). Bianchi et al. (2003) concluded in a survey in New Caledonia that a shorter interval between treatments was significantly associated with a higher level of acaricide resistance in the ticks. In our investigation, a higher number of acaricide treatments per year was significantly associated with multiple resistant ticks. Farmers who reported more than five treatments annually had almost a fourfold increase in the likelihood of having ticks with resistance to the six acaricides tested (Table 4). In regions of southern South America, such as Rio Grande do Sul, where the tick season typically spans 9 months, from September to May (Martins et al., 2002), there is potential to reduce the number of sequential acaricide applications. To this end, generational treatment protocols with the goal to reduce the number of acaricide applications per year have been widely recommended for tick control in this region (Centenaro et al., 2022). Based on our findings, we can propose a practical recommendation for all field practitioners: reducing the

TABLE 4 Multivariate logistic regression analysis of risk factors associated for occurrence of *Rhipicephalus (Boophilus) microplus* resistant to six acaricide compounds in samples of 176 farms from the State of Rio Grande do Sul, Brazil.

Independent variable	Category	*p* value	OR (95% CI)[a]
Region[b]	Southern Region	0.289	-
	Northern Region		
Cattle type production[b]	Beef cattle	0.251	-
	Dairy cattle or mixed cattle		
Cattle type operation	Calf and stocker cattle	0.611	-
	Others		
Cattle breed	European breeds	0.493	-
	Crossbreeds and Indian breeds		
Cattle reposition	External sources (purchase)	0.462	-
	Internal sources		
Cattle herd size[b]	≥232 animals	0.014	10.9 (1.6–73.3)
	<232 animals		
Integrated tick control	Use of integrated strategies (rotational grazing, spelling pasture and/or crop-livestock system)	0.548	-
	No use of integrated strategies		
Injectable formulations[b]	Use of injectable acaricides	0.020	5.5 (1.3–23.2)
	No use of injectable acaricides		
Frequency of acaricide treatments per year[b]	> five acaricide treatments	0.044	3.9 (1.0–14.5)
	≤ five acaricide treatments		
Veterinarian assistance	No veterinarian assistance	0.117	-
	Presence of veterinarian assistance		

Note: Variables statistically associated with resistance to six compounds showed in bioassay.
Abbreviations: 95%CI, 95% confidence interval; OR, odds ratio.
[a]OR were calculated only for variables with *p* values <0.05.
[b]*p* values below 0.05 at multivariate analysis.

number of annual acaricide treatments may help mitigate resistance development.

Ultimately, farms with larger herds (i.e., more than 231 cattle in our study) had more than tenfold increase in the likelihood of having multiple resistant ticks, in the multivariate model. Interestingly, in a study from Ecuador, it was shown that the percentage of annual expenditure on acaricide treatment in relation to the farm budget was proportionally lower for bigger herds, when compared to small farms (Paucar-Quishpe et al., 2023). This suggests that farms with larger herds may have greater financial flexibility to apply more frequent acaricide treatments without significantly affecting their budget, which could, in turn, promote the selection for resistance. These findings should be considered when designing future policies to mitigate the spread of acaricide resistance, as larger herds appear to present a more challenging scenario regarding resistance than smaller ones.

Importantly, all the farmers enrolled in our study were given a report on acaricide resistance on their farms, and they all received a visit from a member of the state veterinary service to aid in understanding of the report and planning further strategies. We consider this study has raised awareness about acaricide resistance in Rio Grande do Sul because several farmers and practitioners contacted our institution about the survey after the beginning of the project. They subsequently sent samples to our institution for routine acaricide resistance diagnosis services. Thus, epidemiological studies and governmental strategies can serve as a stimulus for greater adherence to sustainable practices, such as the routine use of drug resistance diagnosis.

A limitation of our study is that our findings are representative only of large and medium farms (more than 40 animals), which constitute approximately only 20% of all properties in this state. A different cohort of farms (e.g., those with 10–20 cattle) may result in a completely different scenario regarding acaricide resistance. As previously mentioned, acaricide resistance seems to be associated with larger herds. Further efforts should focus on characterising the acaricide resistance scenario on small farms (less than 40 cattle). Additionally, we must consider that only 176 farms were analysed among all 302 farms visited. The remaining farms in which tick collection was not possible may represent sampling bias. In fact, only a low number of engorged female ticks were observed in some farms, which made it impossible to perform bioassays. It is reasonable to hypothesise that farmers experiencing tick control failures due to multiple resistance to acaricides may face higher tick loads, thus favouring their inclusion in the study. The development of molecular tests or novel bioassays using non-engorged ticks and immature stages must be encouraged to address the limitation of tick sampling (Jongejan et al., 2024; Thomas et al., 2020).

Regarding our findings, the issue of multiple resistance is escalating, primarily due to the challenge of developing new strategies to control resistant ticks. Thus, it is crucial to use accurate diagnostic tools to identify populations with multiple resistance to acaricides and mitigate the spread of these ticks, as well as to adopt effective management practices in tick control. Furthermore, urgent actions are needed from all stakeholders involved in cattle breeding, including farmers, veterinarians, the veterinary pharmaceutical industry, and government authorities. These actions should include transmitting information about resistance, diagnostics and tick control; implementing policies to reduce and slow down the potential spread of multiple resistance to acaricides; validating and implementing strategic control programmes, including non-chemical strategies; and safeguarding the use of novel acaricide compounds.

AUTHOR CONTRIBUTIONS

Priscila Teixeira Ferreira: Investigation; writing – original draft; writing – review and editing; formal analysis; data curation; validation. **Nathalia de Bem Bidone:** Conceptualization; project administration. **Fernando Groff:** Conceptualization; funding acquisition. **Patrícia Silva da Silva:** Investigation. **Mariana Silveira de Jesus:** Investigation. **Debora da Cruz Payao Pellegrini:** Formal analysis. **Rovaina Laureano Doyle:** Conceptualization. **José Reck:** Conceptualization; project administration; writing – review and editing; supervision; data curation; formal analysis. **Guilherme Klafke:** Conceptualization; writing – review and editing; project administration; supervision; validation; data curation; formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are included in the article is available in a public repository under the DOI 10.5061/dryad.sj3tx96dd.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Supplementary Table 1. Lethal concentrations and discriminating concentrations for resistance calculated with larvae of *Rhipicephalus (Boophilus) microplus* susceptible reference strain Porto Alegre. The results were obtained with five independent bioassays with each active ingredient. The LCs were calculated by pooling the results of the bioassays.

Supplementary Figure 1. Word cloud showing the most used acaricides in the sampled farms ($n = 176$) from Rio Grande do Sul state, Brazil. The size of each acaricide formulation displayed in the diagram is proportional to the number of times it was cited in the questionnaires. To better show the data, some compounds were abbreviated, as following: cyp, cypermethrin; and chlorp, chlorpyrifos.

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