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Larissa Claudino Ferreira

Avaliação de resistência cruzada e efeito da ciclosporina A em populações de *Rhipicephalus microplus* resistentes a lactonas macrocíclicas no Semiárido Nordestino

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Dissertação submetida ao Programa de Pós-Graduação em Ciência e Saúde Animal, da Universidade Federal de Campina Grande, como requisito parcial para obtenção do grau de Mestre em Ciência e Saúde Animal.

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LARISSA CLAUDINO FERREIRA

AVALIAÇÃO RESISTÊNCIA CRUZADA DE ACARICIDAS PERTENCENTES A CLASSE DAS LACTONAS MACROCÍCLICAS E DETOXIFICAÇÃO METABÓLICA EM POPULAÇÕES DE *Rhipicephalus microplus* NO SEMIÁRIDO NORDESTINO

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RESUMO

O carrapato dos bovinos, *Rhipicephalus microplus*, é o ectoparasita hematófago de maior impacto econômico e sanitário para a bovinocultura do Brasil. O método de controle deste parasito mais utilizado é a aplicação de acaricidas químicos sintéticos, com destaque para as drogas da classe das lactonas macrocíclicas, como ivermectina. Há diferentes mecanismos envolvidos na resistência a antiparasitários, incluindo a atividade aumentada de bombas de efluxo celular como os transportadores de membrana tipo ABC (*ATP binding cassetes)*, que auxiliam na desintoxicação celular dos parasitos, causando resistência às drogas. Este mecanismo de resistência já foi descrito em populações de *R. microplus* resistentes à ivermectina. Pelo fato das lactonas macrocíclicas serem compostos semelhantes, foi investigada a hipótese de existência de resistência cruzada entre diferentes fármacos deste grupo (ivermectina, eprinomectina e moxidectina) e da participação dos transportadores ABC como o mecanismo de resistência envolvido. Foram analisadas quatro populações de *R. microplus* resistentes, provenientes da Região Semiárido do Nordeste do Brasil, dos Estados da Paraíba e do Ceará, sendo duas propriedades de cada Estado. Para isso, foram executados Testes de Imersão Larval com e sem o uso de Ciclosporina A (CsA), um inibidor de transportadores ABC, comparando com a cepa susceptível e de referência Porto Alegre, observando o sinergismo entre CsA e ivermectina, eprinomectina e moxidectina nessas populações. Entre as populações analisadas, foi observada a resistência cruzada entre moxidectina e ivermectina, mas não entre a ivermectina e a eprinomectina. A CsA atuou como sinergista, reduzindo as doses letais de ivermectina e moxidectina, mas não da eprinomectina. Conclui-se que a resistência cruzada entre a ivermectina e a moxidectina pode estar associada à atividade aumentada de atividade de transportadores ABC.

PALAVRAS-CHAVE: carrapatos; acaricidas; ciclosporina a; lactonas macrocíclicas.

ABSTRACT

The cattle tick, *Rhipicephalus microplus*, is the hematophagous ectoparasite with the greatest economic and health impact on Brazilian cattle. The most used method of controlling this parasite is the application of synthetic chemical acaricides, with emphasis on drugs of the macrocyclic lactone class, such as ivermectin. There are different mechanisms involved in antiparasitic resistance, including the increased activity of cellular efflux pumps such as ABC membrane transporters (ATP binding cassettes), which aid in cellular detoxification of parasites, causing drug resistance. This resistance mechanism has already been described in ivermectin-resistant populations of R. microplus. As macrocyclic lactones are similar compounds, we investigated the hypothesis of cross-resistance between different drugs of this group (ivermectin, eprinomectin and moxidectin) and the participation of ABC transporters as the resistance mechanism involved. Four populations of resistant R. microplus were analyzed, from the semi-arid region of Northeast Brazil, from the states of Paraíba and Ceará, with two properties in each state. For this, Larval Immersion Tests were performed with and without the use of Cyclosporine A (CsA), comparing with the susceptible and reference strain Porta Alegre, observing the synergism between CsA and ivermectin, eprinomectin and moxidectin in these populations. Among the populations analyzed, cross-resistance between moxidectin and ivermectin was observed and CsA acted as a synergist, reducing its lethal doses, while for and eprinomectin the results were different from those found for the other macrocyclic lactones used. It is suggested that other resistance mechanisms may be involved for eprinomectin in resistant populations in the semiarid region of Northeastern Brazil.

KEYWORDS: ticks; acaricides; cyclosporine a; macrocyclic lactones.

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LISTA DE ABREVIATURAS E SIGLAS

ABC	ATP-binding cassette
CE	Ceará
CL50	Concentração letal para 50%
CsA	Ciclosporina A
EPM	Eprinomectina
FR	Fator de resistência
FS	Fator de sinergismo
IC95	Intervalos de confiança de 95%
IFPB	Instituto Federal da Paraíba.
IVM	Ivermectina
LM	Lactona macrocíclica
MOX	Moxidectina
PB	Paraíba
POA	Cepa Porto Alegre
TIL	Teste de imersão larval

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INTRODUÇÃO GERAL

O carrapato *Rhipicephalus microplus* é um ectoparasito hematófago que infesta bovinos, sendo responsável por causar grandes prejuízos econômicos, como redução no ganho de peso, e, consequentemente, no rendimento de carcaça, diminuição na produção de leite e desvalorização do couro. Dessa forma, torna-se um dos responsáveis pelas limitações na rentabilidade da bovinocultura brasileira. Estima-se que há uma perda de cerca de 3,24 bilhões de dólares/ano em decorrência do impacto do parasitismo no gado do Brasil (GRISI et al., 2014).

Predominantemente, o controle de *R. microplus* em bovinos é feito através da utilização de produtos químicos. O baixo custo, associado a disponibilidade e fácil aquisição dos produtos, tornam as condições ideais para o desenvolvimento de resistência aos diversos ectoparasiticidas existentes, principalmente ao grupo dos piretróides, organofosforados e das lactonas macrocíclicas, por serem os mais utilizados e os mais conhecidos.

Carrapatos resistentes aos grupos químicos foram descritos em vários Estados do Brasil, ao serem testados acaricidas: no Rio Grande do Sul, ao amitraz, ivermectina, cipermetrina, clorpirifós e fipronil por Klafke et al (2016); múltipla resistência também foi detectada no Mato Grosso do Sul, por Gomes et al. (2011); na Bahia, a organofosforados, piretróides e a suas associações, por Raynal et al. (2018). Na Paraíba, Vilela et al. (2020) encontraram populações de carrapatos resistentes a diversas classes de acaricidas (cipermetrina, clorpirifos, amitraz e ivermectina).

Há acaricidas de aplicação e ação tópica e outros que agem de forma sistêmica, e estes últimos podem ser administrados via *pour on* ou injetável, sendo o ingrediente ativo metabolizado pelo organismo e distribuído pelo sangue do animal hospedeiro, que acaba sendo ingerido pelo carrapato. As lactonas macrocíclicas (avermectinas e milbemicinas) são exemplos de carrapaticidas sistêmicos que revolucionaram o mercado de antiparasitários na década de 1980 por apresentarem

maior poder residual e serem capazes de provocar a morte de parasito internos e externos, e por isso são conhecidos como endectocidas (FURLONG, 2005). São compostos bioativos derivados da fermentação do fungo *Streptomyces avermitilis* (avermectinas) e *Streptomyces cyaneogriseus* (milbemicinas), e o modo de ação envolve canais de cloro dependentes de glutamato e GABA. (BURGE et al., 2006).

Há diversos mecanismos fisiológicos capazes de causar a resistência aos antiparasitários como a resistência metabólica, por alterações estruturais no exoesqueleto ou por alteração de sítio alvo. Outro mecanismo de resistência a drogas é o de desintoxicação por bomba de efluxo, que ocorre por meio dos transportadores ABC (ABCt) sendo esta descrita em bactérias, fungos, endoparasitas e ectoparasitas (POELARENDS et al., 2002; KERBOEUF et al., 2003). De acordo com dados obtidos a partir de experimentos realizados por James e Dayvel (2009) com o nematódeo *Caenorhabiditis elegans,* em um organismo suscetível, o número de ABCt nas células é reduzido e o efluxo da droga não é suficiente para limitar sua eficácia.

O entendimento da resistência a acaricidas pode auxiliar na escolha correta de princípios ativos capazes de controlar infestações por *R. microplus*, assim como, empregar novas técnicas de identificação da resistência, para que se obtenha mais informações acerca dos mecanismos envolvidos na resistência aos acaricidas.

Na região semiárida do nordeste brasileiro, Vilela et al. (2020) observaram uma alta frequência de populações de *R. microplus* resistentes ao clorpirifós, amitraz, cipermetrina e ivermectina, com alto nível de resistência a essa lactona macrocíclica. Por ser de um grupo químico que apresenta componentes semelhantes, tais como a eprinomectina e a moxidectina, pode-se levantar a hipótese do desenvolvimento de resistência cruzada entre estas drogas, determinada pelo mecanismo de desintoxicação por atividade aumentada de ABCt. Assim, o presente trabalho teve por objetivo analisar a ocorrência de resistência cruzada entre os acaricidas ivermectina,

eprinomectina e moxidectina em populações de *R. microplus* resistentes a ivermectina como também analisar se nessas populações ocorre a participação dos transportadores ABC na resistência a esses acaricidas, por meio de bioensaios com o inibidor de ABCt ciclosporina A em populações de carrapatos do semiárido do Nordeste, Brasil.

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CAPÍTULO I:

Effect of cyclosporin A on the toxicity of ivermectin, eprinomectin and moxidectin in populations of *Rhipicephalus microplus*

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Effect of cyclosporin A on the toxicity of ivermectin, eprinomectin and moxidectin in populations of *Rhipicephalus microplus*

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ABSTRACT

Rhipicephalus microplus is mainly controlled through acaricides. However, reports of resistance have become frequent worldwide, including in relation to macrocyclic lactones (MLs). Involvement of ABC transporters (ABCt) in populations resistant to ivermectin has been demonstrated. Thus, the aim of this study was to evaluate the efficacy of ivermectin, eprinomectin and moxidectin with and without use of synergistic cyclosporin A (CsA) in resistant populations of R. microplus using larval immersion tests (LITs). Engorged females were collected from four farms in the semiarid region of northeastern Brazil that had histories of continuous use of ivermectin. Epidemiological questionnaires were applied to collect information about management aimed at controlling ticks on these farms. Resistance to MLs was observed on all of the farms. There was statistically significant synergism ($p < 0.05$) between CsA and ivermectin in all populations; between CsA and eprinomectin in only one population; and between CsA and moxidectin in two populations. It was concluded that, despite the involvement of ABCt in the mechanisms of resistance to ivermectin, metabolic detoxification does not seem to be the mechanism predominantly involved in resistance to eprinomectin and moxidectin in the populations of *R. microplus* evaluated.

Keywords: Ticks, resistance, macrocyclic lactone, synergy.

1 INTRODUCTION

Rhipicephalus microplus is an ectoparasite that affects cattle. In Brazil, its frequency of occurrence varies according to the weather conditions and breeds of cattle raised (Gomes et al., 2011; Raynal et al., 2013; Reck et al., 2014; Torres-Santos et al., 2021). Brazil has approximately 220 million head of cattle (Maia et al., 2014). When these animals are infested by R. microplus, their behavior is affected, as also is their health, directly or indirectly. This implies reduced productivity and great economic losses (Grisi et al., 2002). The main control method against this parasite consists of use of acaricides. However, due to incorrect and exacerbated use, reports of acaricide resistance have been described worldwide (Burrow et al., 2019; Shakya et al., 2020; Vilela et al., 2020; Torres, 2021).

Because resistance is an evolutionary adaptation, the parasite's organism will always try to defend itself from the offending substance. This results in development of behavioral and physiological mechanisms that allow resistant individuals to survive, either through insensitivity of the target site or through increased capacity of the detoxification process (Perry, et al. 2011).

There are several classes of acaricides currently exist. Among these are macrocyclic lactones (MLs), the group to which ivermectin belongs. Since this drug was developed in 1980 (Chabala et al., 1980), it has been among the acaricides most used for controlling cattle ticks. Consequently, cases of resistance to ivermectin are highly frequent (Torrents et al. 2020; Vilela et al. 2020; Valsoni et al. 2021).

Activity of ABC transporters (ABCts) in relation to drug resistance has been described in bacteria, fungi, helminths and ticks (Koenderink et al., 2010; Le Gall et al., 2018; Mate et al., 2022). In susceptible organisms, the number of ABCts in cells is low and the efflux of the drug is insufficient to limit its effectiveness. However, in resistant organisms, the number and/or activity of these transporters increases and the drug is rapidly eliminated from cells or exported into vesicles, which reduces its concentration in the body and impairs its effectiveness (Lespine et al., 2008). This makes it difficult to treat diseases, infections and parasites, mainly because ABCts are related to resistance to multiple drugs, thereby limiting the number of substances that can be used in therapeutic procedures (Lage, 2003; Leslie et al., 2005). Studies carried out by Menéz et al. (2016) on ivermectin-resistant C. elegans demonstrated that ABC transporters are involved in resistance to both ivermectin and moxidectin.

The transcription levels of the Rm-ABCB10 gene are significantly higher in the gut of

blood-fed females in an ivermectin-resistant population. ABCts are responsible for sequestration and detoxification of the heme molecule in the hemosomes present in the intestine of ticks. They are important in detoxification of acaricides, with regard to macrocyclic lactones, in this same organelle (Pohl et al., 2012).

Numerous efforts have been made to improve the techniques for diagnosing resistance and for understanding its mechanisms and how it is genetically transmitted. Based on this knowledge, preventive and control actions against resistance can be taken in a sustainable manner. Using this approach, studies by Pohl et al. (2012) and Khangembam et al. (2018) demonstrated the association between ABCt levels and resistance to ivermectin in R. microplus.

The immunosuppressive drug cyclosporin A (CsA) works as an inhibitor of ABC transporters because it has high affinity for ABC transporters of some classes. Its mechanism of action consists of formation of hydrophobic bonds with them, thus resulting in a low rate of dissociation between them, which determines competitive inhibition (Seelig & Landwojtowicz, 2000).

Therefore, the aim of the present study was to conduct comparative analyses through larval immersion tests (LITs) on the acaricides ivermectin, eprinomectin and moxidectin, with and without addition of the inhibitor cyclosporin A. In this manner, it was sought to evaluate the participation of ABC transporters in resistance to macrocyclic lactones in populations of *R. microplus* in the semiarid region of northeastern Brazil.

MATERIAL AND METHODS

Study sites and information on acaricide use

This study was carried out in the semiarid region of northeastern Brazil. Four cattle-producing farms were visited: two in the state of Paraíba, in the municipalities of São João do Rio do Peixe and Sousa; and two in the state of Ceará, in the municipalities of Várzea Alegre and Barro (Figure 1).

Data on use of acaricides in the populations evaluated were obtained through an epidemiological questionnaire that asked for information about treatments, numbers of animals on the farm and management adopted.

Ticks

The Porto Alegre (POA) isolate was used as a reference susceptible strain. Since it was first isolated, the POA strain has been widely used as a susceptible reference strain (Reck et al., 2014; Vilela et al., 2020).

About 100 engorged females were collected from each farm, comprising pooled ectoparasites from different animals. They were placed in plastic pots with non-airtight lids that allowed air circulation. Subsequently, the material was sent for processing at the Veterinary Parasitology Laboratory of the Federal Institute of Paraíba, Sousa campus. The analysis procedures followed what was described in FAO (2014). After the ticks had been washed in distilled water for approximately two minutes and dried on paper towels, eight to ten of them were placed in Petri dishes for incubation in a BOD (biochemical oxygen demand) environmental chamber, in the dark, at 27 ºC, with relative humidity between 85 and 90%, for 10 to 14 days, to enable egg laying. After this period, the eggs were transferred to 15 ml Falcon tubes, which were closed using a cotton swab to allow passage of air and moisture. The eggs and larvae were incubated under the same conditions as the engorged females. The unfed larvae used in the tests were between 14 and 21 days old.

Macrocyclic lactones and synergists used

Technical grade ivermectin, eprinomectin and moxidectin (Sigma Chemical Co., St. Louis, MO, USA) were used for the bioassays. Cyclosporin A (CsA), a non-ribosomal cyclic peptide that contains eleven amino acids and has several functions, was also used in the bioassays. CsA is widely used in organ transplantation because it has immunosuppressive activity and reduces the possibility of organ rejection (Pubchem®). Studies have shown that CsA can also be used as an inhibitor of ABCt activity, which is involved in the resistance mechanisms observed in relation to ivermectin (Pohl *et al.,* 2011; Le Gall *et al*., 2018).

Preparation of acaricides and use of cyclosporin A

The toxicity of ivermectin, eprinomectin and moxidectin in the presence and absence of cyclosporin A was evaluated through the larval immersion test (LIT), as described by Klafke et al.

(2012). Acaricides were diluted in a solution of 2% Triton X- 100 in absolute ethanol (ETH-TX2%) for eprinomectin and in a solution of 2% Triton X-100 with pure acetone (ACTX-2%) for ivermectin and moxidectin. As a control for all acaricides, a diluent without acaricide was used. Cyclosporin A was added to the acaricide solutions at a constant concentration of 15 μM.

In performing the bioassay, 500 microliters of each immersion solution were distributed into three 1.5 ml microcentrifuge tubes. Using a brush, approximately 100 larvae were transferred to each tube, which was then closed and shaken vigorously to ensure that the larvae subsided. After 10 min of immersion, the larvae were removed from the tube using a clean brush, allowed to dry on a piece of paper towel and then transferred to a filter paper package, which was folded in half and closed on the sides with metal clips. After adding the larvae, the package was sealed with a third clip and incubated in BOD at 27-28 °C and 85–90% relative humidity in the dark. After 24 h, larval mortality was determined by counting the total numbers of live and dead individuals. Larvae that were paralyzed or moving only their appendages, without the ability to walk, were considered dead.

Statistical analysis

Probit analysis was performed on the bioassay results, using the Polo-Plus software (LeOra Software, 2003). For each test, the following parameters were estimated: LC50 (50% lethal concentration) with its 95% confidence intervals (95% CI) and the slope of the regression line. The resistance factor was calculated comparatively, in relation to the susceptible strain (POA), and the LC50 for each field population was determined through LITs, with and without use of cyclosporin A. The synergism factor was calculated from the results by dividing the LC50 acaricide + CsA by the LC50 acaricide without CsA.

RESULTS

The cattle herds ranged in size from 50 to 250 animals. They were predominantly of taurine breeds and had been reared for dairy production. On all farms, there was a history of prolonged use of injectable macrocyclic lactones, together with acaricides of other classes such as synthetic pyrethroids, organophosphates and formamidines, which were applied topically by spraying. In

general, acaricides were administered every eight days to animals in the rainy season of the year (January to May) and every 15 or 30 days in the dry season (June to December).

The LIT results demonstrated the degree of resistance that existed in these populations. For ivermectin, the resistance factor (RF) on the farms ranged from 6.3 to 38.9 (Table 1). In relation to eprinomectin, only the population in Barro, Ceará, showed resistance, with RF 31.4 (Table 2). In relation to moxidectin, all the populations were resistant, with RF ranging from 5.6 to 339.2 (Table 3).

CsA gave rise to synergism with ivermectin in all the four populations evaluated, thus significantly reducing ($p < 0.05$) the LC50 of the drug, with synergism factors (SF) ranging from 1.5 to 3.6 (Table 1). In tests performed using eprinomectin, CsA significantly reduced ($p < 0.05$) the LC50 only in the population of São João do Rio do Peixe, in Paraíba, with a SF of 1.3 (Table 2). Use of CsA demonstrated synergism with moxidectin, thus significantly reducing ($p < 0.05$) the LC50 in the populations of São João do Rio do Peixe, Paraíba, and Barro, Ceará, and showing SF of 1.4 and 2.0, respectively (Table 3).

DISCUSSION

On the farms visited, there were numerous factors that predisposed towards resistance. This included the fact that the majority of the cattle were of taurine breeds that had been reared for dairy production. According to Zangirolamo (2017), animals of this nature are more susceptible to tick infestations. Prolonged, irregular and short-term use of products from different classes of acaricides, in injectable and spray forms, are contributory factors towards selection pressure in these populations. On these farms, ivermectin was the only ML used, and this use gave rise to selection pressure not only on avermectins, but also on milbemycins, which in this study showed the highest RF values.

The RFs for ivermectin in the field populations were high, ranging from 6.3 to 38.9. Lovis et al. (2013) used the susceptible strain Munõz in populations from farms in Argentina, South Africa and Australia, and found lower values that ranged from 0.7 to 6.8. It can be said that the high levels of resistance presented in populations resistant to ivermectin are a reflection of continuous and prolonged use of this macrocyclic lactone, which selects increasingly resistant populations and leads to development of cross- resistance with moxidectin through selection pressure. As can be seen in the values for moxidectin (Table 3), its RF was also extremely high,

ranging from 5.6 to 339.2. On the other hand, for eprinomectin, only farm 3 showed resistance, with a RF of 31.4. Thus, the existence of cross-resistance determined by high use of ivermectin on farms was evident, thus compromising the effectiveness of other MLs. Vermunt et al. (1996) showed that cross-resistance to avermectins and milbemycins can occur in nematodes through continuous use of these molecules.

In LITs performed with ivermectin, addition of CsA gave rise to a significant increase in ML toxicity for all populations (Table 1). Studies carried out by Pohl et al., (2012), LeGall et al. (2016) and Khangembam et al. (2018) on ivermectin and CsA in populations of R. microplus demonstrated that ABC transporters are linked to multidrug resistance. According to James and Davey (2009), from experiments carried out using Caenorhabditis elegans, it was observed that different ABC transporter genes are expressed, depending on the level of resistance of the nematode to ivermectin.

It is known that in resistant populations, when these ABC transporters are overexpressed, they protect cells from the entry of MLs, through limiting their toxic effect (Pohl et al., 2012). As described by Lespine et al. (2008), it is understood that through use of inhibitors such as CsA, the function of ABC transporters is reduced and ivermectin accumulates in cells. Thus, the results obtained suggest that the presence of the inhibitor can promote intracellular accumulation and consequent exacerbation of the toxic effects of ivermectin in ticks, which would otherwise be removed by ABC transporters.

In general, it was not possible to observe any significant increase in the toxicity of eprinomectin and moxidectin when associated with CsA, as observed regarding the association of ivermectin + CsA. Presence of the inhibitor increased the toxicity of eprinomectin only in the population of São João do Rio do Peixe. Paraíba. For moxidectin, there were significant increases in toxicity in the populations of São João do Rio do Peixe, Paraíba, and Barro, Ceará. Although these drugs are analogous and derived from abamectin, it has been suggested that the mechanisms of resistance to eprinomectin (400- epi-acetylamino-400-deoxyavermectin B1) and ivermectin (22,23-dihydroavermectin B1a) are different from each other. Metabolic resistance may have occurred, in which, according to Guerrero et al. (2012) and La Canal et al. (2021), the detoxification capacity of the larvae are increased through the action of the enzymes cytochrome-P450 monooxygenase (P450), esterase and glutathione S-transferase (GST), to eliminate a certain acaricide. This can be explained by the disposition of the carbons, in which in ivermectin there is

hydrogenation of C-22 and C-23 in the synthesis process, while in eprinomectin addition of an acetylamino group at C-4 occurs, with the possibility of divergence in the binding mechanisms, which may be facilitated in ivermectin.

With moxidectin, drug synergism was only significant in populations with higher RFs (339.2 and 22.8), which leads to the hypothesis that ABCt expression may increase as drug resistance rates increase. In the other two populations, in which the synergist had no significant effect, involvement of other detoxification pathways is suggested, as seen for eprinomectin. Thus, these results reflect a difference in selection pressure that developed through continuous use of ivermectin in the population of ticks used. This generates different possibilities for studies on the mechanisms that may be involved in resistance to MLs, such as evaluation of other synergists and analysis of molecular markers and genes involved in resistance.

CONCLUSION

It was concluded that ABC transporters participate in the detoxification process in relation to ivermectin, eprinomectin and moxidectin, in populations of resistant R. microplus in the semiarid region of northeastern Brazil. In part of the populations evaluated for eprinomectin and moxidectin, CsA was effective as an enzyme inhibitor, but in the other parts, other mechanisms associated with resistance are suggested.

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Figure 1. Location of the farms of the field populations used, in the Northeastern semiarid region, in the states of Paraíba and Ceará.

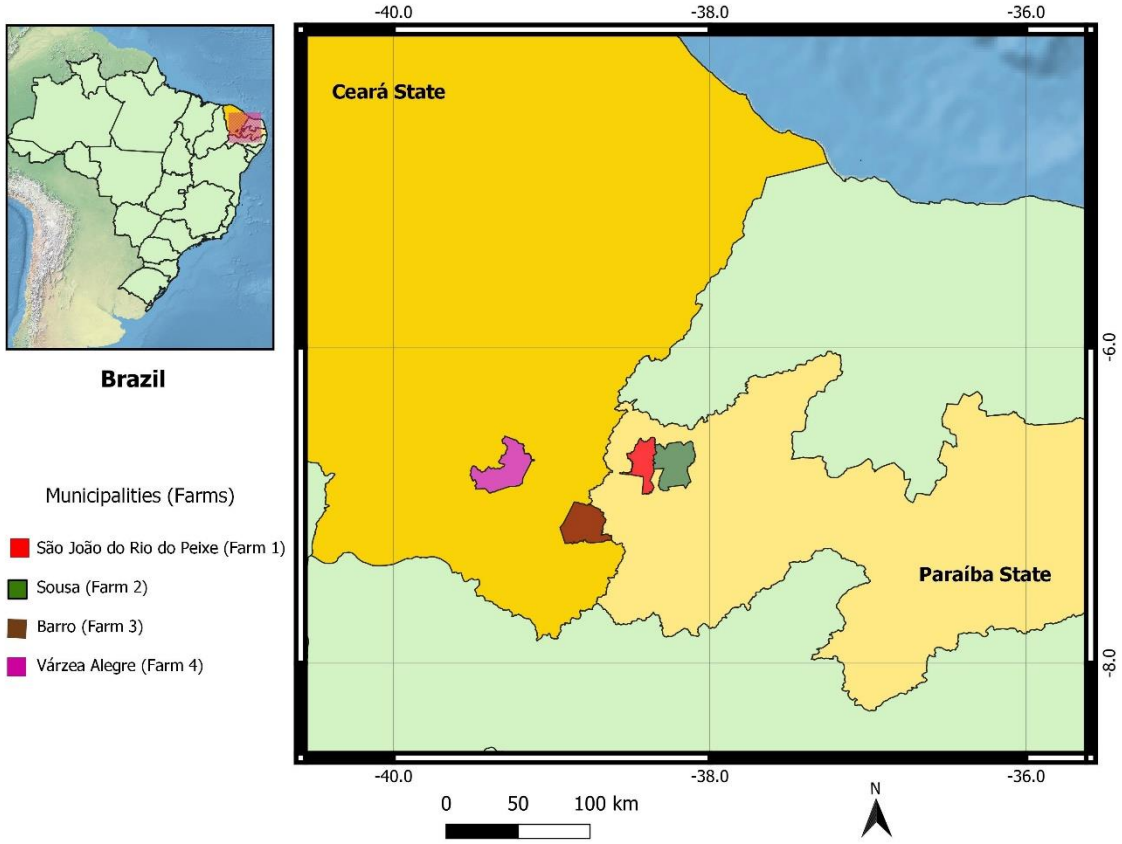

Table 1. Resistance to ivermectin resistance and synergism factors in populations of *Rhipicephalus microplus* in the states of Paraíba and Ceará, Brazil, evaluated with and without use of cyclosporin A, through the larval immersion test (LIT).

Population	**Treatment**	**Nº**	**Inclination (S.E.)**	**LC50 (95% CI)**	**RF**	**SF**
POA	IVM	2087	2.865 (0.154)	26.228 (15.368-39.439)	-	-
	IVM+CsA	2098	7.656 (0.506)	15.860 (14.084-17.699)	-	1.653
1-São João do Rio do Peixe - PB	IVM	1473	1.447 (0.094)	167.214 (146.189-263.727)	6.375	-
	IVM+CsA	2667	1249 (0.044)	87.285 (57.432 -132.995) *	-	1.915
2-Sousa - PB	IVM	3.383	1.305(0.052)	1021.235 (725.243-1525.858)	38.936	-
	IVM+CsA	3.912	1.739(0.057)	280.920 (225.856-347.856) *	-	3.635
3-Barro-CE	IVM	2478	1.105 (0.039)	221.170 (153.254-329.165)	8.432	-
	IVM+CsA	2377	1.163 (0.040)	112.629 (90.851-139.086) *	-	1.964
4-Várzea Alegre-CE	IVM	2.407	3.105(0.212)	527.093 (234.052-782.235)	20.096	-
	IVM+CsA	2.269	3.119(0.261)	332.275 (180.572-446.404)*	-	1.586

S.E.: Standard error. LC50: lethal concentration for 50% of the population; 95% CI: 95% confidence interval; IVM: ivermectin; CsA: cyclosporin A.
RF: resistance factor = LC50 test population / LC50 susceptible reference - POA strain
SF: synergism factor = LC50 ivermectin + CsA / LC50 ivermectin
* asterisks indicate significantly lower LC50 values for ivermectin in the presence of CsA than the LC50 for ivermectin in the absence of CsA, based on overlapping confidence intervals.

Table 2. Resistance to eprinomectin and synergism factors in populations of *Rhipicephalus microplus* in the states of Paraíba and Ceará states, Brazil, evaluated with and without use of cyclosporin A, through the larval immersion test (LIT).

Population	**Treatment**	**Nº**	**Inclination (S.E.)**	**LC50 (95% CI)**	**RF**	**SF**
POA	EPM	2006	3.541 (0.178)	22.439 (15.998-31.032)	-	-
	EPM+CsA	1913	3.177 (0.184)	18.739 (13.947-24.189)	-	1.197
1- São João do Rio do Peixe - PB	EPM	2.306	3.445 (0.177)	5.277 (4.814-5.754)	0.235	-
	EPM +CsA	2.251	1.687 (0.064)	3.810 (2.867-5.057)*	-	1.385
2-Sousa - PB	EPM	1855	0.911 (0.047)	12.434 (7.267-18.950)	0.554	-
	EPM +CsA	1489	0.739 (0.046)	16.599 (11.651-22.483)	-	0.749
3-Barro - CE	EPM	1713	0.958 (0.047)	705.417 (473.761-1141.840)	31.437	-
	EPM +CsA	1892	0.820 (0.042)	710.919 (463.435-1216.963)	-	0.992
4-Várzea Alegre - CE	EPM	1582	0.620 (0.052)	1.308 (0.201-3.711)	0.058	-
	EPM +CsA	1799	0.631 (0.046)	2.333 (0.409-6.187)	-	1.783

S.E.: Standard error. LC50: lethal concentration for 50% of the population; 95% CI: 95% confidence interval; EPM: eprinomectin; CsA: cyclosporin A.
RF: resistance factor = LC50 test population / LC50 susceptible reference - POA strain
SF: synergism factor = LC50 eprinomectin + CsA / LC50 eprinomectin
* asterisks indicate significantly lower LC50 values for eprinomectin in the presence of CsA than the LC50 for eprinomectin in the absence of CsA, based on overlapping confidence intervals.

Table 3. Resistance to moxidectin and synergism factors in populations of *Rhipicephalus microplus* in the states of Paraíba and Ceará states, Brazil, evaluated with and without use of cyclosporin A, through the larval immersion test (LIT).

Population	**Treatment**	**Nº**	**Inclination (S.E.)**	**LC50 (95% CI)**	**RF**	**SF**
POA	MOX	2788	10.454	0.297 (0.281-0.313)	-	-
	MOX +CsA	2624	8.017 (0.624)	0.236 (0.210-0.261)	-	1.258
1-São João do Rio do Peixe – PB	MOX	2.700	1.338 (0.060)	100.748 (65.928-143.057)	339.281	-
	MOX +CsA	2817	1.336 (0.064)	86.989 (43.848-142.360)*	-	1.460
2-Sousa – PB	MOX	3.279	4.985 (0.312)	2.957 (2.712-3.201)	9.956	-
	MOX +CsA	3.136	4.818 (0.314)	4.516 (4.138-4.887)	-	0.654
3-Barro - CE	MOX	2400	7.870 (0.866)	6.798 (5.584-7.731)	22.888	-
	MOX +CsA	2240	3.948 (0.319)	3.353 (2.852-3.811)*	-	2.027
4-Várzea Alegre - CE	MOX	2.288	5.057 (0.272)	1.688 (1.593-1.784)	5.683	-
	MOX +CsA	3.043	2.847 (0.108)	1.601 (1.331-1.927)	-	1.054

S.E.: Standard error. LC50: lethal concentration for 50% of the population; 95% CI: 95% confidence interval; MOX: moxidectin; CsA: cyclosporin A.
RF: resistance factor = LC50 test population / LC50 susceptible reference - POA strain
SF: synergism factor = LC50 moxidectin + CsA / LC50 moxidectin
* asterisks indicate significantly lower LC50 values for moxidectin in the presence of CsA than the LC50 for moxidectin in the absence of CsA, based on overlapping confidence intervals.

CAPÍTULO II:

Cross-resistance between macrocyclic lactones in populations of *Rhipicephalus microplus* in Brazil's semiarid region

Trabalho submetido à revista Experimental and Applied Acarology

Qualis: A1

Cross-resistance between macrocyclic lactones in populations of *Rhipicephalus microplus* in Brazil's semiarid region

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ABSTRACT

Rhipicephalus microplus, also known as the cattle tick, is the parasite with the greatest impact on cattle in Brazil. The most common method for controlling this tick is the application of synthetic chemical acaricides, especially ivermectin, which belongs to the group of macrocyclic lactones (MLs). However, because ivermectin is widely used, there is concern about the development of cross-resistance within this chemical class. Thus, engorged females were collected from farms with a history of resistance to ivermectin, which was the only one among the MLs that was used as an anthelmintic drug. Using the larval immersion test (LIT) technique, bioassays were performed with ivermectin, moxidectin and eprinomectin on populations of *R. microplus* from the semiarid region of the states of Paraíba and Ceará. Epidemiological questionnaires were applied to collect information about tick control management. All the evaluated populations showed cross-resistance between ivermectin and moxidectin, but only one population showed cross-resistance between ivermectin and eprinomectin. Weekly or monthly administration of injectable 1% ivermectin on farms was reported. It was concluded that the frequent use of ivermectin may lead to the development of cross-resistance to moxidectin. For eprinomectin, despite the structural similarity, cross-resistance was not observed in three tick populations.

Keywords: acaricides, cattle tick, eprinomectin, ivermectin, moxidectin.

INTRODUCTION

Rhipicephalus microplus infests cattle in tropical and subtropical regions. Larva, nymphs and adults attach to and develop in a single host, characterizing its life cycle as monoxenous (Taylor, 2019). Although this tick is present throughout the year in Brazil, it reaches its peak in the semiarid region of the country's northeast during the rainy season, which tends to occur from January to May or June (Barros et al., 2017). *R. microplus* is the parasite that has the greatest impact on cattle, causing the transmission of several pathogens, including *Babesia* spp. and *Anaplasma* sp. (Costa et al., 2013). That is why the effective control of this ectoparasite is of utmost concern.

R. microplus populations have developed resistance to all acaricides available on the market (Rodriguez-Vivas et al., 2014; Reck et al., 2014; Villar et al., 2020; Vilela et al., 2020; Valsoni et al., 2020; 2021). The excessive use of acaricides in the absence of knowledge about the life cycle of the arthropod, allied to failures in its detection and mismanagement in animal husbandry, leads to the development of resistance to almost all classes of veterinary drugs on the market, and the more widely these acaricides are applied, the higher the percentage of drug resistance (Kumar et al., 2020; Villareal et al., 2021).

Since its discovery in 1980 (Chabala et al., 1980), ivermectin has been one of the most commonly used drugs in the treatment of *R. microplus* infestations. Ivermectin belongs to the group of macrocyclic lactones (MLs), which is composed of avermectins (ivermectin, eprinomectin and doramectin) and milbemycins (moxidectin). The substances naturally produced by *Streptomyces avermitilis* have been identified as A1a, A1b, A2a, A2b, B1a, B1b, B2a and B2b, with A2a, B1a and B2a being the main products of its fermentation (Brossi, 2018). Avermectin B1 (abamectin) is important because other avermectins are produced from it. Ivermectin (22,23-Dihydroavermectin B1a) is obtained via the hydrogenation of carbon atoms C-22 and C-23 of abamectin, while eprinomectin (400-epi-acetylamino-400-deoxyavermectin B1) is obtained through the addition of the acetylamino group at C-4 (Danaher et al., 2006). Hibbs and Gouaux (2011), as well as Prichard et al. (2012), stated that the composition of ivermectin and moxidectin differs because fermentation occurs through different organisms and they have dissimilar chemical structures. The main structural difference is that avermectins have sugar groups at carbon 13(C13) of the macrocyclic ring, whereas moxidectin has protons at C13. In addition, moxidectin has other differences,

including a methoxime at C23 that can prevent H binding to an M3 loop in the act of drug binding to the receptor, which can cause some molecular shift of this loop. Moreover, the absence of the disaccharide substituent should result in moxidectin lacking two van der Waals binding sites (for M2-M3 loops) for the glutamate (GluCl)-controlled Chlorine Channel of the ectoparasite, thereby possibly causing interactive changes through the difference in methyl to ethyl binding in this position, affecting its power.

MLs act as an analogue of the neurotransmitter γ-aminobutyric acid (GABA) and in GluCl. By binding to chlorine channels, they cause the uninterrupted influx of Cl- ions into neurons at the neuromuscular junction, blocking neurotransmission. Thus, they paralyze the tick's somatic and pharyngeal muscles (Omura, 2008; Klafke, 2011).

The first report of resistance to ivermectin in the tick *R. microplus* (São Gabriel strain) in Brazil was described by Martins and Furlong (2001). Since then, several studies have shown the occurrence of this resistance in different locations (Klafke et al., 2006; 2012; Andreotti, 2010; Lopes et al., 2013; Vilela et al., 2020). In addition, Martins and Furlong (2001) reported cross-resistance between doramectin, moxidectin and ivermectin in studies carried out with the São Gabriel strain. However, the existence of cross-resistance to eprinomectin and moxidectin has not yet been reported in field populations, although ticks showed susceptibility in laboratory experiments (Nazir et al., 2013; Nascimento et al., 2020). However, in studies with moxidectin conducted by Lovis et al. (2013), the population of *R. microplus* on a farm in Argentina proved to be resistant. Nevertheless, the author points out the weakness of her results, as there were no repetitions in the trials. In Brazil, these two compounds are used only occasionally, and the use of ivermectin is more widespread.

Due to the drug resistance of *R. microplus*, particularly against ivermectin, there is a concern about the possibility of cross-resistance between MLs, given their similar molecular structures and mechanisms of action. Therefore, this work aimed to evaluate the existence of cross-resistance between MLs in *R. microplus* populations in the semiarid region of Northeast Brazil, based on in vitro larval bioassays.

MATERIAL AND METHODS

Collection of samples and location of experiments

Samples were collected at four cattle farms in Brazil's northeast semiarid region, covering the municipalities of São João do Rio do Peixe – PB (Farm 1), Sousa – PB (Farm 2), Barro – CE (Farm 3) and Várzea Alegre – CE (Farm 4) (Figure 1). The farms were selected based on their history of use and anthelmintic resistance to ivermectin.

The experimental tests were carried out at the Veterinary Parasitology Laboratory of the Federal Institute of Paraíba – IFPB at the city of Sousa.

Characterization of the farms

In order to build a profile of the farms, a questionnaire was applied containing the following items: bovine population, breed of animals, production system (intensive, extensive or semi-intensive), main purpose (dairy or beef cattle), and size of grazing area. For the management profile for tick control, the following were considered: acaricides applied in the last two years, acaricide applied in the last treatment before ticks were collected, interval between treatments, performance of treatments according to the manufacturers' recommendations, application method (spray, injectable or pour-on formulations), and influence for the choice of a specific acaricide: recommended by a veterinary doctor, veterinary pharmaceutical sales representatives, by neighbors/cooperatives, or determined by product price.

Ticks

The isolate Porto Alegre (POA) was used as a reference susceptible strain. Since it was first isolated, the POA strain has been widely used as a susceptible reference strain (Reck et al., 2014; Vilela et al., 2020).

Approximately 100 engorged females were collected at each farm from cattle that had been treated with topical acaricide at least 30 days earlier, or 45 days after treatment with injectable acaricide, to ensure the absence of interference in the results. Ticks were removed directly from infested cattle and placed in plastic containers (105 mm high, 80 mm in diameter) with holes in the lid to allow the passage of air. The containers were kept in a polystyrene box until their arrival at the Veterinary Parasitology Laboratory (LPV) of the Federal Institute of Paraíba (IFPB) at Sousa.

Ticks were processed at the aforementioned laboratory of IFPB, as specified by FAO

(2004). The ticks were washed in distilled water, dried on paper towels, placed on plastic Petri dishes (90 mm in diameter × 22 mm in height) and incubated in a BOD (Bio-Oxygen Demand) chamber in the dark, at temperatures between 27 and 28 °C, with relative humidity between 85 and 90%. The female ticks were left in the BOD chamber for two weeks, giving them time to lay eggs. Egg masses (250 mg aliquots) were then placed in glass vials (10 mL), which were closed with a cotton lid, allowing air and moisture to pass through. To enable the larvae to hatch, the eggs were incubated under the same conditions as the engorged females.

Acaricides

Technical grade ivermectin, eprinomectin and moxidectin (Sigma Chemical Co., St. Louis, MO, USA) were used.

Larval immersion test (LIT)

Acaricides were diluted in 2% Triton X-100 solution in absolute ethanol (ETH-TX2%) in the case of eprinomectin and 2% Triton X-100 with pure acetone (ACTX-2%) in that of ivermectin and moxidectin dilution. The solutions for dilution were different to ensure a more homogeneous dilution of the acaricides.

Ivermectin, eprinomectin and moxidectin were evaluated with the larval immersion test (LIT), as described by Klafke et al. (2012). A diluent without acaricide was used as a control for all acaricides.

The bioassay was performed using 500 microliters of each immersion solution distributed in three 1.5 ml microcentrifuge tubes. Using a brush, approximately 100 larvae were transferred to each tube, which was then closed and shaken vigorously to ensure the larvae were completely submerged. After 10 min of immersion, the larvae were removed from the tube with a clean brush, allowed to dry on a paper towel, then transferred to a filter paper package folded in half and closed on the sides with metal clips. After adding the larvae, the package was sealed with a third clip and incubated in the dark in an environmental chamber at 27-28 °C and 85–90% relative humidity. After 24 h, larval mortality was determined by counting the total number of live and dead individuals. Larvae that were paralyzed or moving only their appendages, but unable to walk, were considered dead.

Statistical analysis

Probit analysis was performed on the bioassay results, using Polo-Plus software (LeOra Software, 2003). The following parameters were estimated for each test: LC50 (50% lethal concentration) with its 95% confidence interval (95%CI) and the slope of the regression line. Resistance was calculated based on a comparison of the susceptibility of the field population and the susceptible reference strain, determining the LC50 for each field population and the LC50 for the POA strain, in the LIT.

RESULTS

The four visited farms had an average of 272.9 (16.5 – 600) hectares (ha), with areas of native Caatinga pasture and areas with cultivated grasses (*Pennisetum purpureum* and *Cenchrus ciliaris*). The average population of the herds was 123 (52 - 200) cattle. Two farms (1 and 3) are exclusively dairy, while the others have mixed production (beef and dairy). Farms 1 and 3 had Holstein cattle, farm 2 had Holstein and Gyr breeds, and farm 4 had crossbred cattle.

The main health problems reported on the farms were tick infestations (farms 1, 3 and 4), while the main problem reported on farm 2 was the occurrence of bovine parasite sadness. The farms have a history of using sprays and pour-on with synthetic and organophosphate pyrethroids to control ticks. However, the most frequent administration is by injection, with weekly or monthly application of 1% ivermectin to control *R. microplus* and/or *Haematobia irritans*. Producers stated they had never administered eprinomectin and moxidectin against endo- or ectoparasites to their animals. At all the farms, the control of *R. microplus* started when the producers noticed the effect of telegony in the animals.

All the *R. microplus* populations showed resistance to ivermectin when compared to the POA strain. As can be seen in Table 1, ivermectin presented a resistance factor (RF) ranging from 6.3 to 38.9. Only the Barro-CE population demonstrated resistance to eprinomectin (RF= 31.4). Moxidectin presented RF values ranging from 5.6 to 339.2.

All the populations showed cross-resistance between ivermectin and moxidectin. However, only in the Barro-CE population showed collateral resistance between ivermectin, eprinomectin and moxidectin.

DISCUSSION

The farms visited in this study engage in practices that are known to predispose to the development of acaricide resistance. The profiles of the farms indicated that factors such as high animal stocking rate, dairy production and predominance of taurine breeds, as described in the literature (Utech et al., 1978; Veríssimo et al., 2002; Vilela et al., 2002; Vilela et al. al., 2020), were factors that favored the development of parasite drug resistance.

Pereira et al. (2010) reported that inadequate management of acaricides, including incorrect application, continuous use of spray formulations, high frequency of treatments, initiation of treatments only after the discovery of engorged female ticks, and incorrect interval between treatments, facilitate the development of drug resistance. These practices were observed on all the farms that participated in this study.

It was found that ivermectin was the only macrocyclic lactone used on the farms, and that the animals had no contact with eprinomectin and moxidectin. However, resistance to the acaricides ivermectin, eprinomectin and moxidectin was observed in one tick population, and to ivermectin and moxidectin in the other three populations. This is evidence of cross-resistance and underscores the importance of adopting strategies to control the bovine tick in Brazil's semiarid region, mainly due to overuse of macrocyclic lactones.

given their similar structural formulations and mechanisms of action, high cross-resistance between ivermectin and eprinomectin (avermectins) was expected, since they are amino-avermectins derived from Avermectin B1, which have a modified terminal oleandrose moiety called 400-Deoxy-400-epi-acetylamino-avermectin B1. However, cross-resistance between these drugs was only observed in the Barro-CE population. In studies with nematodes, Ménez et al. (2016) demonstrated that with the multidrug-resistant phenotype selected by ivermectin and moxidectin, the nematodes were also more resistant to eprinomectin than the wild type strain, with eprinomectin being the less potent drug after ivermectin and moxidectin selection pressure. Thus, further studies are needed to better characterize the resources the tick *R. microplus* uses to build up its eprinomectin resistance.

Acaricide resistance may be metabolic, via an increase in the detoxification ability of the acaricide; by structural alterations in the exoskeleton, in which the penetration of acaricides is reduced; or molecular, involving target-site mutation, the latter being described in ivermectin-resistant populations (Pohl et al., 2012; Guerrero et al., 2013).

Despite the similarities between macrocyclic lactones, there are important differences between ivermectin and moxidectin, such as plasma and tissue kinetics. Moxidectin can maintain activity for a long period of time (150 days) in different long-acting formulations, such as the drug Cydectin 10% LA (Zoetis, S.A, Belgium). According to Prichard et al. (2012), when resistance to avermectins first develops, it usually influences the effectiveness of all avermectins. However, as a milbemycin, moxidectin is generally still highly effective against avermectin-resistant parasites at its recommended dose rate. According to Ranjan et al. (2002), parasite resistance to moxidectin develops more slowly than to ivermectin. However, the findings of this study suggest that resistance to moxidectin may occur as a result of continuous selection pressure through the use of ivermectin.

Observing and comparing the RF results of the tick populations, it can be stated that there are different types of receptors and/or existing connections. These differences in the responses to ivermectin and moxidectin suggest that there are differences in the level of interaction of GluCl with these molecules, or that the involvement of other amino-restricted chlorine channels with the actions of avermectins and moxidectin differ, as suggested by Lespine et al. (2012) in their studies of nematodes.

CONCLUSIONS

In view of the findings of this study, it can be stated that *R. microplus* populations from Brazil's northeastern semiarid region exhibit collateral resistance between the macrocyclic lactones ivermectin and moxidectin, which may be caused by selection pressure, favored by the long-term use of ivermectin. However, although eprinomectin is structurally similar to ivermectin, this type of resistance was not observed.

Statements

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Conflict of interest statement

The authors declare that they have no conflict of interest relevant to the content of this article.

Ethical approval

The activities involved in this research were approved by the Ethics Committee for Animal Use of our institution (CEUA/IFPB), under protocol number 23000.000558.2021-23.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Code availability

Not applicable.

Author contributions

All the authors contributed to the conception and design of the study. Material preparation, data collection and analysis were carried out by Larissa Claudino Ferreira, Estefany Ferreira, Ana Luzia Peixoto, Clarisse Silva de Menezes Oliveira, Geraldo Ribeiro, Luana Carneiro de Sousa, Guilherme Marcondes Klafke, Thaís Ferreira Feitosa, Vinícius Longo Ribeiro Vilela, Caio Márcio de Oliveira Monteiro. The first version of the manuscript was written by Larissa C. Ferreira, Guilherme M. Klafke and Vinícius Longo R. Vilela; and all the authors made comments in previous versions of the manuscript. All the authors read and approved the final manuscript.

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Table 1. Cross-resistance status of *Rhipicephalus microplus* populations in the states of Paraíba and Ceará, Brazil, using the Larval Immersion Test (LIT).

Farm	Population	Treatment	Nº	Slope (S.E.)	LC_{50}(IC95%)	RF
-	POA	IVM	2.087	2.865 (0.154)	26.228 (15.368-39.439)	-
		EPM	2.006	3.541 (0.178)	22.439 (15.998 – 31.032)	-
		MOX	2.788	10.454	0.297 (0.281 – 0.313)	-
1	São João do Rio do Peixe - PB	IVM	1473	1.447 (0.094)	167.214 (146.189-263.727)	6.375
		EPM	2.306	3.445 (0.177)	5.277 (4.814 – 5.754)	0.235
		MOX	2.700	1.338 (0.060)	100.748 (65.928 – 143.057)	339.281
2	Sousa-PB	IVM	3.383	1.305(0.052)	1021.235 (725.243-1525.858)	38.936
		EPM	1.855	0.911 (0.047)	12.434 (7.267 – 18.950)	0.554
		MOX	3.297	4.985 (0.312)	2.957 (2.712 – 3.201)	9.956
3	Barro - CE	IVM	2478	1.105 (0.039)	221.170 (153.254-329.165)	8.432
		EPM	1.713	0.958 (0.047)	705.417 (473.761 – 1141.840)	31.437
		MOX	2.400	7.870 (0.866)	6.789 (5.584 – 7.731)	22.888
4	Várzea Alegre-CE	IVM	2.407	3.105(0.212)	527.093 (234.052-782.235)	20.096
		EPM	1.582	0.620 (0.052)	1.308 (0.201 – 3.711)	0.058
		MOX	2.288	5.057 (0.272)	1.688 (1.593 – 1.784)	5.683

S.E.: Standard error; LC50: lethal concentration for 50% of the population; CI 95%: 95% confidence interval; IVM: ivermectin; EPM: eprinomectin; MOX: moxidectin.

RF: resistance factor = LC50 test population / LC50 susceptible reference strain POA

CONCLUSÃO GERAL

Conclui-se que em populações de *R. microplus* do Semiárido Nordestino com histórico de uso da ivermectina a longo prazo, existe uma resistência cruzada entre as lactonas macrocíclicas ivermectina e moxidectina. Enquanto que com a eprinomectina, apesar de apresentar-se mais parecida estruturalmente com a ivermectina, não pode ser observada esse tipo de resistência. Além disso, através da utilização da ciclosporina A pode-se concluir que há a participação dos transportadores ABC no processo de detoxificação à ivermectina em populações de *R. microplus* resistentes no Semiárido do Nordeste do Brasil, e que, com eprinomectina e moxidectina essa eficácia não foi observada em todas as propriedades, levando a hipótese de que ocorra a detoxificação dos fármacos através de outros mecanismos, como a resistência metabólica.