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Syringe immersion test as *in vitro* bioassay against *Rhipicephalus microplus*: Macrocyclic lactones dose-response relationship

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Abstract

Background: For the diagnosis of tick sensitivity against different acaricides, there are *in vitro* and *in vivo* methods. The main *in vivo* method, the stable test, is considered a defining methodology. In Uruguay, the *Rhipicephalus microplus* (*R. microplus*) strain Mozo is used as the standard susceptible strain by the regulatory authorities. *In vitro* techniques applied both on adult and larvae stages are validated by FAO and can serve as an orientation diagnosis of the resistance profile developed in field conditions. An alternative was proposed as a modification of the larval immersion test (LIT), where syringes were used seeking to reduce the work necessary to perform the original technique, resulting in the syringe immersion test (SIT).

Aim: The aim of this study was to expand the SIT for the characterization of sensitivity to Macrocyclic Lactones (MLs) in *R. microplus* and provide information on field strain sensitivity of *R. microplus* larvae.

Methods: Log-logistic dose-response model for Ivermectin (IVM), Doramectin (DRM), and Moxidectin (MOX) were performed using concentrations ranging from 0.01 to 20.0 ppm (n = 6, 3 replicates per level on each drug). Larvae sensitivity results were determined after 24 hours of incubation at 27°C/90% RH, counting live/dead larvae. The final model will be decided as the best fit according to the model selection AIC criteria for each drug. Pharmacodynamic parameters [lower limit, slope, and effective dose at different levels (ED_{20}, ED_{50}, ED_{80}, and ED_{95})] and its 95% confidence interval were considered for drug comparison.

Results: Dose-response models were fitted for IVM, DRM, and MOX. MOX had the lowest ED50 of the three drugs, implying that MOX is of higher potency (two folds) when compared to IVM and DRM on *R. microplus* larvae using SIT. DRM had a different slope compared to IVM and MOX ($p < 0.05$), while IVM and MOX showed a similar slope ($p > 0.05$).

Conclusion: This study allowed us to standardize the technique for larvae immersion for each ML, granting a new tool for *in vitro* test as a screening technique for tick sensitivity.

Keywords: Drugs, Ectoparasiticides, Pharmacodynamics, Resistance, Ticks.

Introduction

Rhipicephalus microplus (*R. microplus*) is one of the most destructive ectoparasites of livestock in tropical and subtropical areas. They are responsible for severe economic losses through both the direct effects of blood feeding and indirectly as vectors of pathogens (Eckstein *et al*., 2015; Molento, 2020). Annual losses due to tick infestation of *R. microplus* were estimated to be US$3.24 billion (Grisi *et al*., 2014) and US$573.61 million (Rodriguez-Vivas *et al*., 2017) for Brazil and Mexico, respectively.

As stated by Rajput *et al*. (2006), ticks must be controlled to meet the world's needs for animal protein. The main methods of control can be classified into chemical methods (application of acaricides) and non-chemical methods (application of biological products and biological control). Biological control can be established using natural predators, such as the cattle egret (*Bubulcus ibis*) or parasites (*Escherichia colli*, *Cedecea lapagei,* and *Enterobacter agglomerans*) (Gonzalez, 1975; Brum, 1988) or even applying fungi as *Metarhizium anisopliae*, as discussed by Da Costa *et al*. (2002). Another control method is based on the application of natural products made from plant extracts (Guerra, 1985), especially when considering nontarget organisms and environmental effects (Panella *et al*., 2005; Dietrich *et al*., 2006).

Currently, chemical treatments are almost the only available resource for the control of this parasite (Fiel and Nari, 2013), with several disadvantages due to the

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high cost of products and damage caused by residues (for nontarget organisms and the environments) (Pruett, 1999). Cattle tick control has traditionally been based on the application of acaricide strategies by dip bath, spraying, pour-on, or injection (Cruz *et al*., 2021). Incorrect dilutions, inappropriate application, overdosing, and/or persistent use are among the main factors that contribute to the emergence of acaricide resistance in ticks (Obaid *et al*., 2022), with growing concern for residues in milk and meat subproducts, affecting the public health through the contamination of the food chain (Camargo-Mathías, 2018). The predominant contributing factors in the development of resistance may include misuse of drugs (Bianchi *et al*., 2003) and use of the wrong concentration of acaricide (Dolan, 1999), leading to the failure of the tick control program (Pegram *et al*., 2000).

There are different methodologies to determine the efficacy and efficiency (FAO, 2004; Holdsworth *et al*., 2022—Appendix A) of acaricides against *R. microplus*. For the *in-vitro* techniques, both adult engorged females [adult immersion test, AIT (Whitnall and Bradford, 1947)] or larvae can be used [larval package test, LPT (Stone and Haydock, 1962); larval immersion test, LIT (Shaw, 1966)]. According to Klafke *et al.* (2012), LIT is a technique based on the immersion of tick larvae in different solutions to later let dry and be placed on a packet of filter paper folded by the middle and then closed on the sides with metal clips, showing similarities with the LPT. The LPT presented lower sensitivity than LIT, which is a more sensitive test, detecting resistant phenotypes in a population even when present at a low frequency, assisting in the early diagnosis of resistance in the field.

Sindhu *et al*. (2012) proposed a modification of the LIT technique using syringes to reduce the labor required to perform the original technique, leading to the emergence of the syringe immersion test (SIT). Farias *et al*. (2016) compared the SIT to the original LIT, concluding that the SIT proposed by Sindhu *et al*. (2012) presented various advantages such as: a) reduction of syringe preparation time, b) reduction of the physical space needed to store the larvae during the test, c) reduction of the risk of environmental and operator contamination with the solutions used (due to lower volume and improvement in the drying process), and d) reduction of the volume of solution used.

Macrocyclic lactones (MLs) are divided into two main groups: avermectins and milbemycins. The avermectins used as ectoparasiticides are ivermectin (IVM), abamectin, and doramectin (DRM); among the milbemycins are milbemycin and moxidectin (MOX). These drugs act as high-affinity agonists on the α-subunit of chloride-selective ion channels present in the parasite. In previous experiments using the Sao Gabriel strain, Martins and Furlong (2001) demonstrated cross-resistance between DRM, MOX, and IVM. It is necessary to have a sensitivity diagnostic tool that allows obtaining results in the shortest possible time from a low number of adult ticks or from larvae of *R. microplus* populations of the different establishments that wish to establish or update their control or eradication plan in the field. The aim of this study was to expand the SIT for the characterization of Mozo strain sensitivity to ML and provide information on the field strain sensitivity of *R. microplus* larvae between IVM, DRM, and MOX.

Materials and Methods

Chemicals

IVM (Lot 49450511) and DRM (Lot 07492109) were donated by Compañia Cibeles S.A. (Uruguay). MOX (Lot MX-A2007025) was obtained from Laboratorio Pasteur S.A. (Uruguay). All other reagents used in this work were from SIGMA Chemical Company. Stock solutions were prepared in acetone [IVM (2,000 ppm), DRM (2,000 ppm), and MOX (100 ppm)].

R. microplus larvae

Mozo strain

In Uruguay, the *R. microplus* strain Mozo is used as the standard susceptible strain by the regulatory authorities. The larvae are obtained after the incubation of teleogynous ticks according to the specifications indicated by Drummond *et al*. (1973). In brief, adult female ticks are conditioned in Petri dishes and incubated with controlled temperature and humidity (27°C and 90%). After 14 days of incubation, the xenogyns are removed, and a new 25-day incubation period is continued for the hatching of viable larvae. Once hatching has occurred, 14–16 days are waited to begin larval testing. This point allows the comparison of different populations of larvae synchronized at the same time of development in terms of vitality and survival time.

Field strain

Larvae were obtained from three engorged ticks from each of four different animals and stored individually (n = 12) to assess for drug sensitivity variance among the field strain. Adult ticks, eggs, and larvae were managed by applying the same protocols as *Mozo* strain. The farm manager reported reduced efficacy when using IVM for *R. microplus* control.

Syringe assembly for SIT

2.5 mL syringes were used, to which the pivot was cut, and a 120 µm filter mesh was adjusted. The syringes are connected to the vacuum pump, and by adding tips, only viable larvae (those that show active movement) are captured. Once the syringes are loaded, holes are made to allow air entry at the time of immersion. The plunger is placed, covering the holes to prevent larvae from escaping.

Larvae immersion

The immersion of larvae was based on the publication of Chaparro-Gutiérrez *et al*. (2020). Syringes loaded with larvae are subjected to immersion for 5 minutes, in the different Drug:Diluent solution prepared from

each stock solution. Corresponding drug dilutions were prepared daily according to the methodology published by FAO (2004). The diluent was used as a control solution, formulated from 1% acetone, and 0.02% Triton-X in distilled water. After the immersion time, syringes are removed, dried on drying paper, and placed in a flow hood for 1 hour prior to incubation for 24 hours. Incubation conditions are similar to those established for adult ticks (27°C and 90% relative humidity). After 24 hours, larval mortality was determined by counting both live and dead larvae. Larvae that were paralyzed or that moved only their appendages, but were unable to walk, were considered dead.

Mozo strain

For IVM and DRM, the final concentrations used to adjust the dose-response model were in the range of 20–0.155 ppm, and in the case of MOX, the final concentrations were between 1 and 0.01 ppm. Larvae immersion was tested in triplicate. A total of six dose-response curves were adjusted for every drug.

Field strain

From the models fitted to the Mozo strain, the field strain was tested at the ED_{50}, ED_{80}, and ED_{95} concentrations estimated for IVM, DRM, and MOX.

Pharmacodynamic function

Larvae mortality and log-transformed concentration data were used to assess the pharmacodynamic profile for increasing acaricides concentrations (four-parameter log-logistic model) (Ritz *et al*., 2015). The maximum effect (Emax) for each curve was set to 1 (100% mortality), being that the maximum possible mortality cannot exceed 100%. The estimated values for the lower limit of efficacy, slope (indicating the sensitivity of the technique), ED_{50} (as an estimation of the potency), and ED_{95} (considered as a discriminating dose) are shown along with their standard error. Statistical significance was determined with a 95% confidence interval.

Statistical analysis

The dose-response models were fitted for the Mozo strain using R software (R Core Team, 2023) and the *drc* package (Ritz *et al*., 2015). The ratio for the pharmacodynamic parameters (lower, slope, and ED_{50}) was calculated between IVM, DRM, and MOX. Kruskal-Wallis tests were performed for the field strain at ED_{50}, ED_{80}, and ED95 for each drug. All tests were performed with a statistical significance set to 95%.

Ethical approval

Not needed for this study.

Results

Dose-response fitting

Model fitting for larval mortality of each drug (IVM, DRM, and MOX) and the pharmacodynamic parameters are shown in Figure 1 and Table 1, respectively. For each drug, dose-response models are summarized, considering the six adjusted curves as one.

Pharmacodynamic parameters comparison

The ratio for the pharmacodynamic parameters (lower limit, slope, and ED_{50}) is shown in Table 2. The ED_{50} ratio between the drugs included in this study showed a significant difference between IVM and DRM when compared to MOX; a similar situation appears when comparing the lower limit. ED_{50} ratio between IVM and DRM versus MOX is higher than 1, meaning that MOX has the lowest ED_{50} of the three drugs, implying that MOX is of higher potency (two folds) when compared to IVM and DRM on *R. microplus* larvae, using SIT. In contrast, DRM has a different slope compared to IVM and MOX ($p < 0.05$), while IVM and MOX showed a similar slope ($p > 0.05$). This would indicate that all drugs achieve maximal efficacy, with DRM having the lowest sensitivity relative to IVM and MOX in modifying its response from minimal to maximal efficacy.

SIT tested on field populations

Individual efficacy results for each field tick, and Mozo strain by SIT using IVM, DRM, or MOX in field populations are shown in Figure 2. At the three levels studied (ED_{50}, ED_{80}, and ED_{95}), all field ticks showed individual efficacies lower than those expected with the reference strain (*Mozo*). Although with ED_{95}, the response was clearly lower than expected (all values were lower than 50% efficacy), it is here where a greater dispersion of individual response was visualized with a range of efficacy variation from two-fold (IVM and DRM) to ten-fold (MOX) between ticks. For the ED_{50} and ED_{80} levels, the low sensitivity of the field strains to each drug did not allow for distinguishing differences in their efficacies (all samples were below 20% mortality).

Discussion

SIT is presented as an alternative to diagnose *R. microplus* susceptibility to MLs. Sensitivity tests performed on adult ticks (AIT) have logistical disadvantages compared to techniques performed on larvae (Spickett *et al*., 1983). Among the disadvantages of using adult individuals, Jonsson *et al*. (2007) stated that these tests require a larger number of individuals to increase the number of replicates and increase the predictive value of the test. Obtaining a sufficient number of adult ticks to perform the sensitivity diagnosis could be difficult in certain epidemiological periods or particular situations of infestation within a farm. Another disadvantage that occurs when working with adult ticks is that the vitality of the individuals is affected by the time elapsed from capture to implementation of *in-vitro* assays (Klafke *et al*., 2012); the opposite case occurs when using larvae, where there is a time window between the capture of adults ticks in the field and larvae hatching from eggs. Using a biological matrix such as *R. microplus* larvae, we were able to increase the number of replicates per individual obtained to include in the analysis, ensuring, on the one hand, a greater number of replicates and, on the other, evaluating the response in the offspring of the ticks already present in the animals. Both aspects are

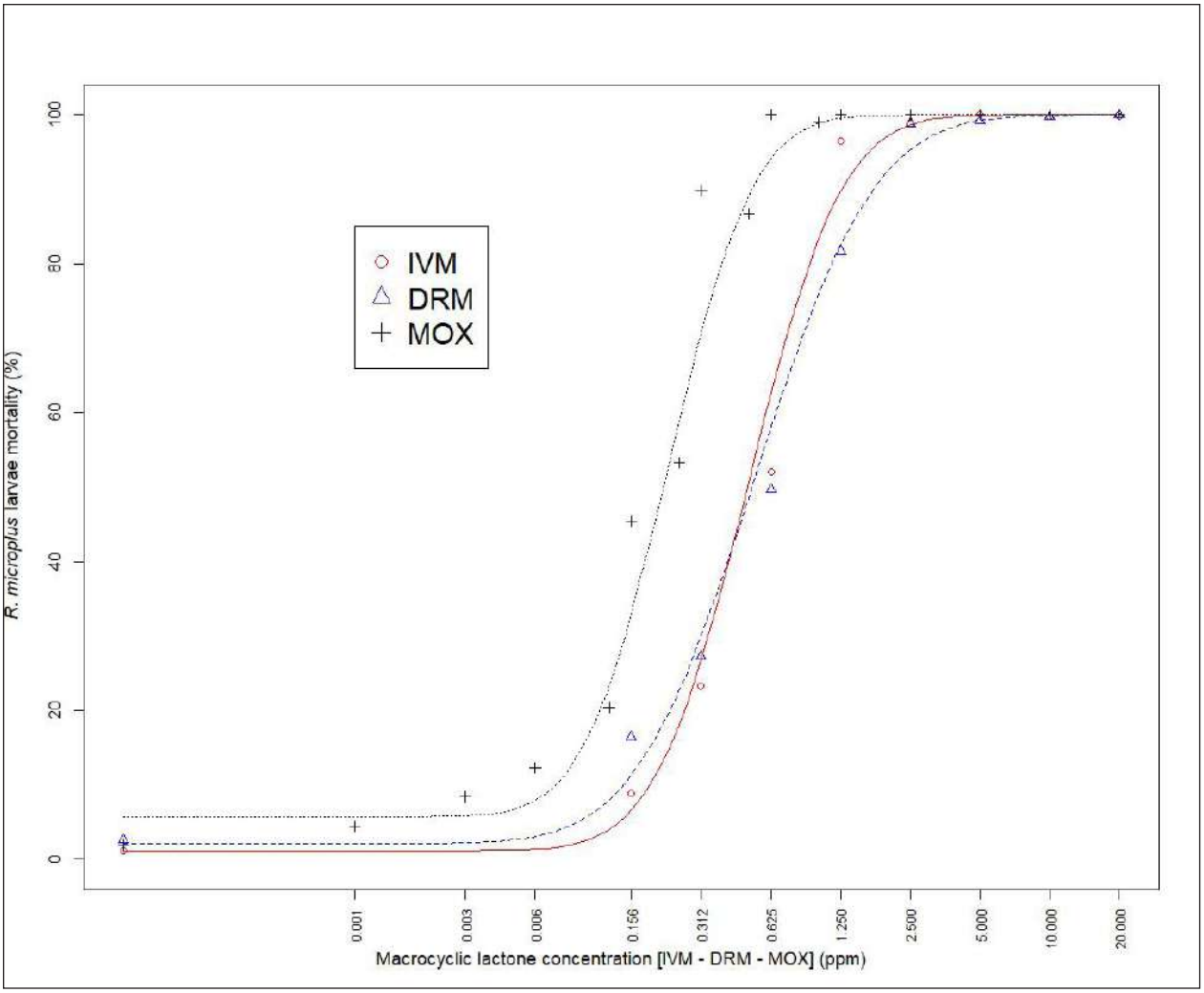

Fig. 1. Dose-response model fitting for *R. microplus* Mozo strain larvae mortality against IVM, DRM, and MOX using SIT

Table 1. Pharmacodynamic parameters for dose-response model on *R. microplus Mozo* strain larvae against IVM, DRM, and MOX using SIT.

	Lower (ppm) [CI_{95}]	Slope (ppm) [CI_{95}]	Effective dose (ppm) (ED) [CI_{95}]			
			ED_{20}	ED_{50}	ED_{80}	ED_{95}
IVM	0.01 [0.0–0.02]	1.37 [1.26–1.49]	0.27 [0.25–0.3]	0.5 [0.47–0.53]	0.92 [0.85–0.99]	1.65 [1.48–1.83]
DRM	0.02 [0.01–0.04]	1.07 [0.00–1.16]	0.24 [0.22–0.27]	0.53 [0.49–0.57]	1.16 [1.07–1.26]	2.45 [2.15–2.76]
MOX	0.06 [0.04–0.08]	1.51 [1.34–1.68]	0.13 [0.11–0.14]	0.23 [0.21–0.24]	0.39 [0.37–0.42]	0.67 [0.6–0.75]

Table 2. Pharmacodynamic parameters ratio [lower limit, slope, and effective dose 50 (ED_{50})] between dose-response models on *R. microplus* Mozo larvae against IVM, DRM, and MOX using the SIT.

Parameter	Comparison	Estimate ± Std error (ppm)	*p*-value
Lower	IVM/DRM	0.57 ± 0.34	0.21
	IVM/MOX	0.21 ± 0.11	0.00
	DRM/MOX	0.37 ± 0.14	0.00
Slope	IVM/DRM	1.28 ± 0.08	0.00
	IVM/MOX	0.91 ± 0.07	0.18
	DRM/MOX	0.71 ± 0.05	0.00
ED50	IVM/DRM	0.94 ± 0.05	0.22
	IVM/MOX	2.21 ± 0.11	0.00
	DRM/MOX	2.35 ± 0.13	0.00

relevant for obtaining reliable and predictive results in the use of drugs for parasite control.

Differential aspects to consider when performing larval sensitivity techniques (LIT *vs*. SIT) are the immersion time and larval handling. Regarding the immersion time, Sabatini *et al*. (2001) stated that the toxicity of adult females to IVM is positively influenced by the immersion time. Klafke *et al.* (2012) evaluated different immersion times for AIT (1, 5, and 30 minutes); for the mentioned authors, the advantage of using 30 minutes of immersion lies in the lower use of active ingredients, although the results obtained present a higher variability, probably due to the lower number of replicates compared to lower immersion times. For techniques using larvae (LPT and LIT) Klafke *et al*. (2012) compared both, obtaining dissimilar results for the parameters of lethal concentration 50 (LC_{50}), where the toxicity of IVM using LPT was lower than for LIT. The LC_{50} of LPT was 90 times higher than the LC_{50} obtained by LIT (1236 *vs.* 16 ppm, LPT and LIT, respectively).

Using larval immersion techniques, the whole individual is exposed to the concentration, ensuring drug entry via cuticular and joint routes, potentially increasing drug entry to the parasite, which could explain the higher sensitivity of the immersion techniques. In our model for IVM, after fitting the values to the log-normal scale for the six dose-response curves studied, the ED_{50} ranged from 0.47 to 0.53, while the slope was between 1.26 and 1.49. In comparison with that reported by Klafke *et al.* (2012) for LPT and LIT, our results obtained with SIT are markedly below those obtained by different techniques. This allows us to indicate that SIT could be an alternative technique that would provide greater sensitivity for the early detection of field strains resistant to IVM.

Regarding larval manipulation, vigorous shaking of larvae during LIT is described by Klafke *et al*. (2012), along with constant manipulation of individuals. Employing SIT avoids the mechanical effect of vigorous shaking, ensuring complete immersion of larvae within the syringe while reducing larval manipulation, both potential sources of variation found in LPT and LIT. In our assay, using the same IVM-sensitive *Mozo* strain, we expanded the range of concentrations needed for the construction of dose-response curves to obtain a better characterization of the response (20–0.156 ppm).

Our study extends the validation of the SIT technique to other MLs (DRM and MOX). Regarding DRM and MOX, *in-vitro* tests on *R. microplus* intermediate life stages are scarce, being mainly used for field efficacy tests based on commercial formulations. The efficacy profiles of these acaricides are focused on field studies on adult forms of the cattle tick. Brito *et al*. (2011) worked with different families of acaricides on field strains (106 commercial establishments). In the case of MLs, they compared *in-vitro* efficacy on adult ticks of the genus *R. microplus*, using commercial formulations diluted in distilled water until the desired concentrations were achieved (10 ppm for the AIT technique), obtaining efficacy results above 95% for IVM, DRM, and MXD, for all field strains evaluated.

Potency (ED_{50}) ratio analysis between avermectins (IVM and DRM) showed no differences against *R. microplus* larvae ($p > 0.05$). However, when analyzing the data for the 95% effective dose (ED_{95}), a marked difference between the concentration needed to achieve an ED_{95} is evident, where DRM requires lower concentrations to achieve the mentioned effect in a sensitive population. This may be due to the differences in the estimated values for the slope of the dose-response curve for the three endectocidal molecules. DRM presents a steeper (lower) slope compared to IVM (1.07 for DRM and 1.37 for IVM), reflecting differences in the speed of changes for *R. microplus* larvae of the Mozo strain when the SIT technique is used, where changes in the concentrations of both DRM and IVM generate increases in larval mortality but DRM does so gradually, requiring higher concentrations to reach the larval mortality levels of IVM. On the other hand, MOX presents a similar slope

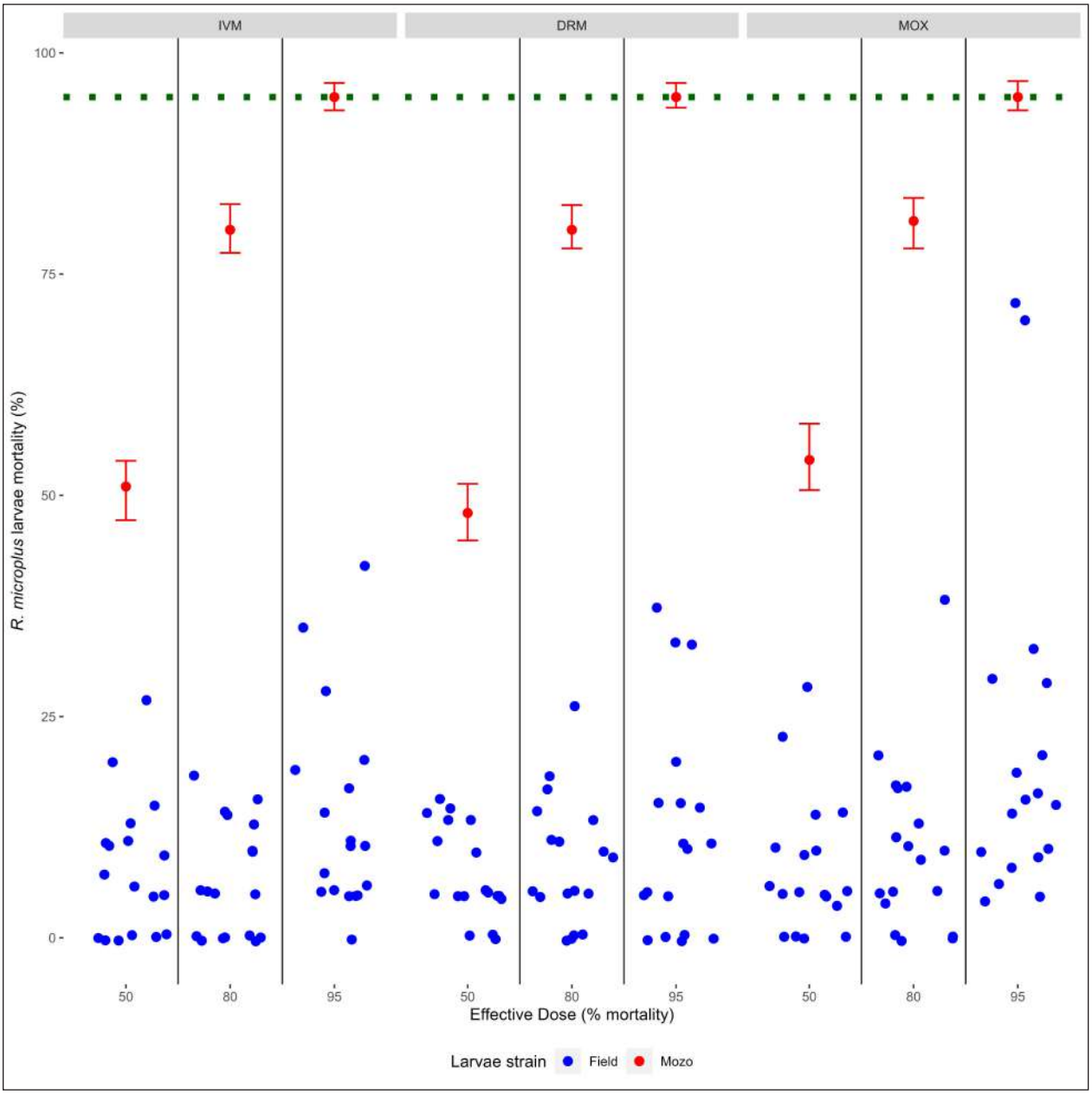

Fig. 2. Individual efficacy results for *R. microplus* on Mozo strain and field population larvae, by SIT on IVM, DRM, and MOX.

to IVM but with higher potency ($ED_{50MOX} < ED_{50IVM} = ED_{50DRM}$), achieving the same level of effect at lower concentrations. A possible explanation could be that MOX has a higher lipophilicity than IVM ($logP_{MOX} = 6$; $logP_{IVM} = 4.8$) (Ménez *et al*., 2012[a]), which would favor the penetration of the acaricide upon contact with the larvae during immersion, resulting in higher mortality at lower concentrations. Prichard *et al*. (2012) stated the structural differences between avermectins (IVM and DRM) and milbemycins (MOX) and how they impact both pharmacokinetic and pharmacodynamic behavior, where the interaction of MOX with glutamate-mediated chloride receptors (mechanism of action of MLs) differs from IVM, suggesting differential involvement of other amino-gated chloride channels with the actions of avermectins and MOX. Within the variety of ligand-mediated chloride receptors, at some receptors, IVM was more potent than MOX, whereas at other receptors, MOX was more potent than IVM (Prichard *et al*., 2012).

When using field strains, larval mortality against IVM, DRM, and MOX was found below the mortality thresholds when compared with the Mozo strain, either when comparing by drug or by ED level within

each drug (ED_{50} *vs.* ED_{80} *vs.* ED_{95}), indicating the possibility of a field strain developing resistance genes against MLs. Acaricide resistance may be metabolic (increasing the detoxification ability of the acaricide), structural alterations in the exoskeleton (reducing the penetration of the acaricides), or molecular (target-site mutation) (Pohl *et al*., 2012). *R. microplus* shows widespread resistance against MLs, particularly against IVM, leading to a concern about the possibility of cross-resistance between MLs, given their similar molecular structures and mechanisms of action (Ferreira *et al*., 2022). The emergence of resistance to any avermectin reduces efficacy against all avermectins, while milbemycins maintain high efficacy against avermectin-resistant populations (Ferreira *et al*., 2022), being that resistance to milbemycins develops more slowly than to avermectins (Ranjan *et al*., 2002). Although, in our work, we only evaluated toxicological response, results are indicative of cross-resistance between MLs, considering that the ticks came from animals that received IVM as a parasitological control drug. Similar conclusions were made by other authors (Pohl *et al*., 2012) regarding the presence of cross-resistance between IVM and MOX in field strains from commercial farms where MOX had never been used for ecto or endoparasite control.

Conclusion

The SIT for *R. microplus* larvae is presented as an alternative for the determination of the sensitivity of tick strains in cattle against IVM, DRM, and MOX, being a new monitoring tool for the sensitivity of *R. microplus* to MLs in integrated parasite control programs.

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Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors contributions

GS and DR designed this study and performed the data analysis. JC performed the laboratory tests. GS and DR wrote and edited the manuscript. JC edited the manuscript. GS and DR finalized the manuscript, and all the authors approved the final version.

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Data availability

The data that support the findings of this study are available from the authors upon reasonable request.

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