Successive treatments with ivermectin (3.15%) to control the tick Rhipicephalus (Boophilus) microplus in cattle: pharmacokinetic and efficacy assessment

Macarena Sarli , María Victoria Miró , María Victoria Rossner , Santiago Nava , Adrián Lifschitz

PII: S1877-959X(21)00201-6
DOI: https://doi.org/10.1016/j.ttbdis.2021.101848
Reference: TTBDIS 101848

To appear in: *Ticks and Tick-borne Diseases*

Received date: 1 June 2021
Revised date: 24 August 2021
Accepted date: 11 September 2021

Please cite this article as: Macarena Sarli , María Victoria Miró , María Victoria Rossner , Santiago Nava , Adrián Lifschitz , Successive treatments with ivermectin (3.15%) to control the tick Rhipicephalus (Boophilus) microplus in cattle: pharmacokinetic and efficacy assessment, *Ticks and Tick-borne Diseases* (2021), doi: https://doi.org/10.1016/j.ttbdis.2021.101848

Successive treatments with ivermectin (3.15%) to control the tick *Rhipicephalus (Boophilus) microplus* in cattle: pharmacokinetic and efficacy assessment

Macarena Sarli[a*], María Victoria Miró[b], María Victoria Rossner[c], Santiago Nava[a], Adrián Lifschitz[b]

[a]Instituto de Investigación de la Cadena Láctea (IdICaL) (INTA-CONICET), Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela (INTA E.E.A. Rafaela), Ruta 34 Km 227, CP 2300, Rafaela, Santa Fe, Argentina

[b]Laboratorio de Farmacología, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET-CICPBA, Facultad de Ciencias Veterinarias, UNCPBA, CP 7000, Tandil, Prov. de Buenos Aires, Argentina

[c]Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Colonia Benítez (INTA E.E.A. Colonia Benítez), Colonia Benítez, Chaco, Argentina

*Corresponding author: sarli.macarena@inta.gob.ar (M. Sarli)

Abstract

This study aimed to evaluate the pharmacokinetics, the potential accumulation in the body of treated animals and the efficacy of ivermectin long-acting formulation (3.15%) against the cattle tick *Rhipicephalus (Boophilus) microplus* in a scheme of three successive treatments. Fifteen 12-month-old heifers, naturally infested with *R. microplus*, were divided into two groups (G). Cattle from GI (n=10) were subjected to three treatments with ivermectin 3.15% (IVOMEC GOLD®, Merial Argentina S.A.) at a rate of 1 mL/50 kg on days 0, 35, and 70. Cattle from GII (n=5) were not treated. From day 1 to 202 post-treatment blood samples were taken to measured ivermectin concentrations by HPLC and female ticks (4.5-8 mm) were counted to evaluate the efficacy of the treatment. The level of tick resistance to ivermectin was evaluated before and after finishing the scheme of successive treatments by larval immersion test (LIT) bioassay from engorged females collected from GI. The area under the concentration vs. time curves ($AUC_{0\text{-}35d}$) obtained post-second treatment was 1.51 ± 0.39-fold higher than those observed post-first treatment ($P<0.05$). The mean plasma concentrations of ivermectin 3.15% at 20 days after the first, second and third treatment were 17.0, 27.5 and 37.8 ng/mL, respectively ($P<0.01$). The elimination half-life of ivermectin post-third treatment was significantly longer than that was previously reported after a single dose ($P<0.01$). Values of therapeutic efficacy percentage reached 75.6% post-first treatment and between 95.9 and 100% after the second treatment. Ticks evaluated by LIT showed a significant increase in lethal concentrations after treatments. Although the efficacy level was high, the successive treatments with long-acting ivermectin formulation generate a significant accumulation of drug in

plasma and could increase the levels of resistance to this drug in the tick population.

Keywords: *Rhipicephalus microplus*, ivermectin 3.15%, therapeutic efficacy, drug residues, pharmacokinetics

1. Introduction

The cattle tick *Rhipicephalus (Boophilus) microplu*s is one of the most important pest of cattle in tropical and subtropical areas worldwide due to its impact on animal production (Jongejan and Uilenberg, 2004). *Rhipicephalus microplus* generates depression of milk production and weight gain, mortality, hide damage and morbidity. In addition, the costs of its control and the effects related to tick-transmitted hemoparasites (*Babesia bigemina, Babesia bovis* and *Anaplasma marginale*) must be added (Spath et al., 1994; Reck et al., 2014). Control of *R. microplus* infestations on cattle is almost entirely based on the applications of synthetic chemical acaricides, but this method has drawbacks as multidrug resistance and accumulation of chemical residues in meat and milk (George et al., 2008; Guerrero et al., 2012; Klafke et al., 2017).

Ivermectin is an endectocide belonging to the class of macrocyclic lactones and is characterized by high efficacy against endo- and ecto-parasites and the long persistence of the antiparasitic activity (Campbell and Benz, 1984). The use of long-acting formulations of ivermectin (3.15%) to control *R. microplus* infestation on cattle is widespread. In Argentina, there are several commercial formulations of ivermectin 3.15% officially approved for the control of this tick (see http://www.senasa.gob.ar). However, the emergence of *R. microplus* populations resistant to ivermectin (Rodríguez-Vivas et al., 2014; Klafke et al., 2017; Torrents et al., 2020) and the long withdrawal period of the long-acting commercial formulations (50 to more than 100 days) constitute a constraint for these pharmaceutical preparations.

The use of treatment schemes based on successive applications of ivermectin 3.15% may result in a reduction of the tick refuge, which could lead to the development of resistance. For example, if three successive applications of most of the different commercial formulations of ivermectin 3.15% approved for control-ticks in Argentina are made with an interval of 35 days, ticks belonging to different generations could be exposed to the treatment (see the population dynamics-patterns of *R. microplus* in Argentina in Canevari et al. (2017) and Nava et al. (2020)). Furthermore, successive applications of the drug could have an additive effect on the accumulation of residues in cattle, extending the withdrawal period. Nava et al. (2019) observed that the application of two successive treatments with ivermectin 3.15% may increase its accumulation in cattle tissues. The knowledge about how the application of successive treatments with ivermectin 3.15% determines the pharmacokinetic patterns of the drug and its efficacy is fundamental for an appropriate design of control methods for *R. microplus.* Therefore, this work aimed to: I) study the pharmacokinetics of ivermectin 3.15% in a scheme of successive treatments and the potential accumulation in the body of treated animals; II) evaluate the efficacy of three successive treatments with ivermectin 3.15%.

2. Materials and methods

2.1. Experimental animals, treatments and sampling

Fifteen 12-month-old Braford heifers were used as experimental animals in the Estación Experimental Agropecuaria Colonia Benítez, Instituto Nacional de Tecnología Agropecuaria (INTA EEA Colonia Benítez), Colonia Benítez (27°20′S, 58°56′W), Chaco Province, north- eastern Argentina. These heifers, naturally infested with *R. microplus,* were divided into two groups. Cattle belonging to Group I (n=10) were subjected to three treatments with a subcutaneous injection of a commercial formulation of ivermectin 3.15% (IVOMEC GOLD®, Merial Argentina S.A.; lot# 88164/19, expiration date 05/2022) at a rate of 1 mL/50 kg of body weight (630 µg/kg) on day 0 (8th January 2020), 35 (12th February 2020) and 70 (18th March 2020) (Fig. 1). The interval between treatments was determined based on the residual effectiveness for absolute tick control (23 days in the case of IVOMEC GOLD®) plus a period of 12 days. Cattle did not receive other antiparasitic treatments and they did not interact with other animals until the end of the trial (November 2020). Cattle belonging to Group II (control group) (n=5) were not treated. Blood samples were taken from the jugular vein from all the treated animals into heparinized tubes (Fig.1). The blood was subsequently centrifuged for 10 min at 750 x *g* and 24 ºC to separate the plasma, which was frozen at −20 ºC until processing for high-performance liquid chromatography (HPLC) analysis (Section 2.2). For practical purposes, in the rest of this text the days post-treatment will be expressed according to the last treatment applied and only if necessary will they be expressed in another way. Handling of animals was made in accordance with the institutional guide for the care and use of

experimental animals, with the approval of the Institutional Committee for Care and Use of Experimental Animals, CICUAE-INTA, Argentina (Council resolution number P21-025).

2.2. Pharmacokinetic study

Ivermectin concentrations in plasma samples were analyzed following the methodology described by Lifschitz et al. (1999, 2000) .The ivermectin chemical extraction process used an aliquot of 0.25 mL of plasma and 1 mL of acetonitrile (J.T. Baker®, Center Valley, PA, USA) as solvent. Moxidectin (Sigma-Aldrich, USA) was included in each sample as internal standard. The mixture of samples and the solvent were shaken (Multi Tube Vortexer, VWR Scientific Products, West Chester, PA, USA) for 20 min (2400 rpm) and then samples were sonicated (ultrasonic frequency kHz 35) for 10 min (Transsonic 570/H, Laboratory Line Instruments Inc., Melrose Park, IL, USA). Finally, the samples were centrifuged at 2000 x *g* for 15 min and the supernatant was recovered in the khan tubes. The supernatant was concentrated to dryness under a stream of nitrogen. The dry residue of ivermectin was derivatized as previously described by de Montigny et al. (1990).

Ivermectin concentrations were determined by HPLC using a Shimadzu 10A HPLC system with an autosampler (Shimadzu Corporation, Kyoto, Japan). HPLC analysis was performed using a reverse phase C18 column (Kromasil, Eka Chemicals, Bohus, Sweden, 5 µm, 4.6×250mm) and a mobile phase of 0.2% acetic acid in water/methanol/acetonitrile (1.6/60/38.4) at a flow rate of 1.5 mL/min at 30 ºC (Lifschitz et al., 1999). Ivermectin was detected with a fluorescence detector (Shimadzu, RF-10 Spectrofluorometric detector, Kyoto, Japan), reading at 365 nm (excitation) and 475 nm (emission wavelength).

Linearity was established to determine the ivermectin concentrations/detector responses relationship. Calibration curves were prepared within the 0.5–100 ng/mL range. Calibration curves were established using least squares linear regression analysis and correlation coefficients (r) and coefficient of variations (CV) were calculated. Mean percentage of ivermectin absolute recovery from plasma were >78.4%. The method precision measured with the coefficient of variation was between 3.16 and 9.27%. The limit of quantification was established at 0.5 ng/mL.

2.3. Pharmacokinetic analyses

A non-compartmental pharmacokinetic analysis was performed with the PK Solutions 2.0 computer software (Ashland, Ohio, USA). The peak concentration (C_{max}) was read from the plotted concentration-time curve in each animal. The area under the concentration vs. time curves (AUC) was calculated by the trapezoidal rule (Gibaldi and Perrier, 1982). The plasma concentrations and systemic exposure calculated as AUC of ivermectin after the successive treatments were simulated using PCModfit 6.9 software (Allen, 1990). The estimated values were compared to those observed after ivermectin quantification in plasma for each treatment. The terminal (elimination) half-life ($T_{1/2}$ el) was calculated as $In2/\lambda_z$, where In2 is the natural logarithm of 2 and λ_z, the slope of the terminal phase. The λ_z was determined performing regression analysis using six points of the terminal phase of the concentration-time plot. Plasma concentrations and the pharmacokinetic parameters are reported as mean ± standard deviation (SD). Mean pharmacokinetic parameters were statistically compared using Student´s *t* test or ANOVA. Significant differences among SD were subjected to a non-parametric Mann Whitney test or Kruskal

Wallis test. The statistical analyses were performed using the Instat 3.0 software (GraphPad Software, CA, US).

2.4. Efficacy assessment

Counts of *R. microplus* females (4.5–8.0 mm long) were performed on the left side of each bovine and the whole tail between days 14 and 202 post-first treatment (see details in Fig. 1). This is due to the fact that on day 0, animals from Group I had a low abundance of countable ticks, with presence of immature stages and that animals from Group II were incorporated to the trial after day 10 post-first treatment due to logistic reasons. The number of half-body counted ticks was multiplied by two for statistical analyses. Prevalence (number of infested hosts / number of examined hosts) and median with first and third quartiles (1Q–3Q) were calculated. Data were subjected to the Shapiro-Wilk's test of normality prior to statistical analysis. Because the test revealed significant deviations from the normal distribution, statistically significant differences in the distributions of *R. microplus* numbers between groups were determined by using the non-parametric Mann-Whitney test. Differences were considered significant at $P<0.01$. Therapeutic efficacy percentage was calculated with the Abbot's formula using the mean number of ticks (Abbott, 1925): Corrected % = (1- n in T after treatment / n in Co after treatment)*100, where n is the mean number of ticks, T is the treated group (Group I) and Co is the untreated group (Group II).

2.5. Larval immersion test for ivermectin resistance in ticks

To determine the level of tick resistance to ivermectin by *in vitro* tests, engorged females were obtained from cattle belonging to Group I before and after finishing the scheme of successive treatments. The progeny of these

female ticks (14-21 days old larvae) were used in the *in vitro* tests. The larval immersion test (LIT) bioassay with ivermectin was performed as described in Klafke et al. (2012) and Torrents et al. (2020), by using technical grade ivermectin (22,23-dihydroavermectin B1, batch number MKCK0618, Sigma-Aldrich, USA). Briefly, an initial solution prepared with technical ivermectin, 1% acetone, Triton X-100 and distilled water was used to prepare 10 mL of the following solutions (in ppm) 4, 5.8, 8.2, 11, 16.8, 32.4, 43.96, 51.4, and 100. The control solution consisted of diluent without ivermectin. Approximately 100 larvae were exposed during 10 min to 1 mL of each solution in 1.5 mL microcentrifuge tubes.

Mortality data were analyzed through a probit analysis with POLO PLUS software (LeOra Software, 2003). Lethal concentrations (LC) for 50% and 99% with their confidence intervals (CI) and the slope of the regression line were estimated by means of a regression equation analysis to the probit transformed data. LC_{50} and LC_{99} values obtained for the post-treatment sample were compared with those values of the pre-treatment sample. Differences between LC values of tick samples were considered significant when its 95% CI_s did not overlap. Resistance ratios (RR) were determined considering both LC_{50} and LC_{99} values as follow: LCx of post-treatment sample / LCx of pre-treatment sample. The criterion considered a priori to interpret the results obtained with the LIT bioassay is based on that established by Castro-Janer et al. (2011): -Susceptible: LC_{50} (CI 95%) of the post-treatment sample is not statistically different from the susceptible strain considered as reference (pre-treatment sample); -Incipient resistance: LC_{50} (CI 95%) of the post-treatment sample is statistically different from the susceptible strain considered as reference and

RR_{50} is <2; -Resistant: LC_{50} (CI 95%) of the post-treatment sample is statistically different from the susceptible strain considered as reference and RR_{50} is ≥ 2.

3. Results

Mean ivermectin 3.15% plasma concentrations measured in cattle after the three treatments are shown in Fig. 2. After the first treatment, there was an increase from day 1 (mean value 8.78 ng/mL) to day 7 (mean value 27.5 ng/mL) and then a decrease to day 35 post-first treatment when cattle received the second treatment. After the administration of the second and third treatment of ivermectin, a significant drug accumulation was observed. The $AUC_{0\text{-}35d}$ obtained post-second treatment was 1.51 ± 0.39-fold higher than those observed post-first treatment ($P<0.05$). The mean plasma concentrations of ivermectin on day 20 post-treatment were significantly different ($P<0.01$) after the administration of the three successive treatments. Whereas post-first treatment drug plasma level was 17.0 ng/mL, it was 27.5 and 37.8 post-second and third treatment, respectively. The observed ivermectin accumulation in the experimental animals after the successive treatments agreed to that estimated by pharmacokinetic simulation (Fig. 3). This simulation predicted the same degree of accumulation that those observed in the field, being the AUC and the C_{max} 57 and 48% higher, respectively, after the third treatment than after the first treatment.

The elimination half-life of ivermectin was calculated from the day 20 post-third treatment with the long-acting formulation. The mean value was 42 ± 11 days, that resulted significantly longer than the values previously reported for the same formulation administered in a single dose (16.5 ± 0.70 days) ($P<0.01$) (Lifschitz et al., 2007).

The therapeutic efficacy of the successive applications of ivermectin 3.15% is shown in Table 1, where data of mean number of ticks, median, prevalence and therapeutic efficacy percentage are presented. Group I and Group II showed similar tick infestation on day 14 post-first treatment (P>0.01). Differences in tick infestation between the two groups were statistically significant in all counts (P<0.01) from day 20 post-first treatment (Table 1). From day 20 post-first treatment, the number of ticks in cattle from Group I remained low throughout the entire trial. Tick abundance in animals without treatment (Group II) showed a natural variation over time, but its decrease was not enough to affect significantly the calculation of efficacy percentage. Values of therapeutic efficacy percentage reached 75.6% post-first treatment and between 95.9 and 100% after the second treatment (Table 1). There was a negative correlation (r= 0.651; P= 0.034) between the ivermectin systemic exposure measured as AUC and the cumulative number of ticks on day 70 post-first treatment when the therapeutic efficacy was 100% (Fig. 4). The cumulative number of ticks in cattle from Group I was significantly lesser than that from the controls (1158 and 3226, respectively) (P<0.01).

The results obtained by LIT *in vitro* bioassay to test the evolution of the level of resistance of ticks after application of successive treatments with ivermectin 3.15% are showed in Table 2. The samples of ticks evaluated in pre and post-treatments periods showed significant differences in the values of LC_{50} and LC_{99}. The values of LC_{50} increased from 8.73 ppm (pre-treatment sample) to 10.5 ppm (post-treatment sample). According to the criteria described in “materials and methods”, the sample post-treatment is classified as “incipient resistance” (RR_{50} 1.2).

4. Discussion

The pharmacokinetics and efficacy of three successive treatments with a long-acting formulation of ivermectin 3.15% to control the cattle tick *R. microplus* was evaluated in this work. A significant accumulation in plasma of ivermectin 3.15% was observed after two and three successive treatments. The ratio of exposure accumulation was between 1.51 and 1.57 after the second and the third treatment. These results agreed with a previous study performed by Nava et al. (2019) where accumulation of ivermectin 3.15% in plasma and fat after two successive treatments with a long-acting formulation was observed. An important issue is related to the potential influence of drug accumulation on the established withdrawal period. Ivermectin is a very lipophilic drug that is widely distributed from the blood to different tissues including fat which may act as drug reservoir (Lanusse et al., 1997, Lifschitz et al., 2000). The pharmacological rationale of the long-acting formulations is based on the vehicle innovation that favors a slow absorption from the subcutaneous site and prolongs the persistence of ivermectin concentrations (Lifschitz et al., 2007). However, the potential advantage of long-acting ivermectin formulation addressed to extend the period of drug protection and to reduce the labor costs of farmers. The withdrawal period of IVOMEC GOLD® is 122 days and the accumulation ratio measured in the current trial was slightly higher than that obtained after the successive treatments with a chemical formulation with a withdrawal period of 55 days (1.42) (Nava et al., 2019). The elimination half-life of ivermectin after the third treatment in the current trial was significantly longer compared to the values previously reported after a single treatment with the same formulation (Lifschitz et al., 2007). The withdrawal time is influenced by

the terminal half-life and therefore, an estimation of this period may be done using the tissue tolerance and the initial drug concentration (Riviere and Sundlof, 2009). The main target tissues for residues evaluation in the case of ivermectin are liver and fat (Chiu and Liu, 1989). Therefore, if the fat tolerance adopted by European Union is used (100 ng/g) (EMA, 2014), the ivermectin accumulation and longer elimination half-life observed after the successive treatments may prolong the withdrawal period beyond 120 days. On the other hand, using the higher tolerance established by FAO for fat tissue (400 ng/g) (FAO, 2016) and recently adopted in Argentina, the accumulation of ivermectin should not prolong the withdrawal period. Although this estimation is theoretical, it is useful to gain a perspective on what the withdrawal time may be altered by pharmacokinetic modifications.

The therapeutic efficacy of three successive treatments with ivermectin 3.15% against *R. microplus* achieved high values of efficacy. The therapeutic efficacy values on day 20 and 35 post-first treatment were 75.6 and 74, respectively, but the therapeutic efficacy values obtained from day 20 post-second treatment (day 55 post-first treatment) were always higher than 95%, reaching values of 100% between days 35 post-second treatment and 49 post-third treatment (days 70 and 119 post-first treatment, respectively) (Table 1). These results coincide with previous studies performed with a single dose of ivermectin 3.15% which failed to achieve 100% of therapeutic efficacy (Lopes et al., 2013; Nava et al., 2019; Torrents et al., 2020). As drug uptake by ticks after the subcutaneous treatment is related to the feeding habits, the systemic exposure measured as AUC is relevant for obtaining therapeutic concentrations (Lifschitz et al., 2007). In fact, there was a negative correlation between

ivermectin systemic exposure and cumulative number of ticks on day 70 post-first treatment.

Although this scheme based on successive treatments with ivermectin 3.15% allows obtaining high levels of therapeutic efficacy, it implicates long periods of exposure of the tick population to the same drug. The concept of refuge refers to the part of the parasite population untreated and thus, free from the selection pressure applied by exposure to drug (Hodgkinson et al., 2019). Therefore, three successive applications of a long-acting acaricide, as the commercial formulations of ivermectin 3.15%, could reduce the refuge in the pasture and increase the selection pressure by eliminating susceptible individuals from the population. This selection could be enhanced as the elimination half-life of the drug was prolonged due to the repeated administration. The selection of individuals carrying resistance genes that are inherited by subsequent generations can affect the efficacy of the drug. The few ticks that were observed on cattle between days 35 post-first treatment and 20 post-second treatment (day 55 post-first treatment) (from early February to early March) could correspond to resistant specimens that were not affected by the treatment. According to the seasonal dynamics of the non-parasitic phase described by Nava et al. (2020) for the same study area, the progeny of those ticks observed on the treated cattle between February and March could correspond to the ticks observed on cattle in late May and June (see Table 1). The results obtained with the LIT bioassay suggest that the *R. microplus* ticks collected after the application of three successive treatments with ivermectin 3.15% could have increased levels of resistance to this drug. These results

empirically show the high risk of increasing the selection pressure for resistance when a tick population is exposed for long periods to a same long-acting drug.

5. Conclusions

The treatment with long-acting formulations of ivermectin (3.15%) based on successive applications in order to suppress or reduce the population of *R. microplus* is a common practice in livestock establishments from Argentina. The results of this study showed that, although the efficacy level of this scheme of treatments to control ticks was high, it has drawbacks as the significant accumulation of drug in plasma and the potential increase in the selection pressure for resistance on the tick population.

Acknowledgement

This work was supported by INTA (PE-E5-I109), Asociación Cooperadora INTA Rafaela, Asociación Cooperadora INTA Colonia Benítez, Agencia Nacional de Promoción Científica y Tecnológica (PICT-2015-550; PICT-2018-0830), and Ministerio de Ciencia, Tecnología e Innovación Productiva de la Provincia de Santa Fe (PIO140).

Declaration of interest

The authors declare no conflicts of interest.

References

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Am. Mosq. Control Assoc. 3, 302–303. https://doi.org/10.1093/jee/18.2.265a.

Allen, G.D., 1990. Modfit: a pharmacokinetics computer program. Biopharm Drug Dispos. 11, 477-498. https://doi.org/10.1002/bdd.2510110603.

Campbell, W.C., Benz, G.W., 1984. Ivermectin: a review of efficacy and safety. J. Vet. Pharmacol. Ther. 7, 1–16. https://doi.org/10.1111/j.1365-2885.1984.tb00872.x.

Canevari, J.T., Mangold, A.J., Guglielmone, A.A., Nava, S., 2017. Population dynamics of the cattle tick *Rhipicephalus (Boophilus) microplus* in a subtropical subhumid region of Argentina for use in the design of control strategies. Med. Vet. Entomol. 31, 6–14. https://doi.org/10.1111/mve.12199.

Castro-Janer, E., Rifran, L., González, P., Niell, C., Piaggio, J., Gil, A., Schumaker, T.T.S., 2011. Determination of the susceptibility of *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) to ivermectin and fipronil by larval inmersion test (LIT) in Uruguay. Vet. Parasitol. 178, 148-155. https://doi.org/10.1016/j.vetpar.2010.12.035.

Chiu, S.H.L., Lu, A.Y.H., 1989. Metabolism and Tissue Residues. In: Campbell W.C. (Eds.), Ivermectin and Abamectin. Springer, New York, NY, pp. 131-143. https://doi.org/10.1007/978-1-4612-3626-9_8.

de Montigny, P., Shim, J.S.K., Pivnichny, J. V., 1990. Liquid chromatographic determination of ivermectin in animal plasma with trifluoroacetic anhydride and N-methylimidazole as the derivatization reagent. J. Pharm. Biomed. Anal. 8, 507–511. https://doi.org/10.1016/0731-7085(90)80060-3.

EMA, 2014. European Public MRL Assessment Report (EPMAR): Ivermectin. EMA/CVMP/ 294840/2014.

FAO, 2016. Ivermectin Residue Monograph. Residue Evaluation of Certain Veterinary Drugs. Joint FAO/WHO Expert Committee on Food Additives (JECFA), 81st meeting 2015. FAO JECFA Monographs 18.

Gibaldi, M., Perrier, D., 1982. Pharmacokinetics. In: Revised and Expanded, 2nd edn. Marcel Dekker, Inc., New York, USA.

George, J.E., Pound, J.M., Davey, R.B., 2008. Acaricides for controlling ticks on cattle and the problem of acaricide resistance. In A. Bowman and P. Nuttall (Eds.), Ticks: Biology, Disease and Control. Cambridge: Cambridge University Press, pp. 408-423. https://doi.org/10.1017/CBO9780511551802.019

Guerrero, F.D., Lovis, L., Martins, J.R., 2012. Mecanismos de resistência aos acaricidas em *Rhipicephalus (Boophilus) microplus*. Rev. Bras. Parasitol. Vet. 21, 1–6. https://doi.org/10.1590/S1984-29612012000100002.

Hodgkinson, J.E., Kaplan, R.M., Kenyon, F., Morgan, E.R., Park, A.W., Paterson, S., Babayan, S.A., Beesley, N.J., Britton, C., Chaudhry, U., Doyle, S.R., Ezenwa, V.O., Fenton, A., Howell, S.B., Laing, R., Mable, B.K., Matthews, L., McIntyre, J., Milne, C.E., Morrison, T.A., Prentice, J.C., Sargison, N.D., Williams, D.J.L., Wolstenholme, A.J., Devaney, E., 2019. Refugia and anthelmintic resistance: Concepts and challenges. Int. J. Parasitol. Drugs Drug Resist. 10, 51-57. https://doi.org/10.1016/j.ijpddr.2019.05.001.

Jongejan, F., Uilenberg, G., 2004. The global importance of ticks. Parasitology. 129, 1–12. https://doi:10.1017/S0031182004005967.

Klafke, G.M., Castro-Janer, E., Mendes, M.C., Namindome, A., Schumaker, T.T.S., 2012. Applicability of in vitro bioassays for the diagnosis of ivermectin resistance in *Rhipicephalus microplus* (Acari: Ixodidae). Vet. Parasitol. 184, 212–220. https://doi.org/10.1016/j.vetpar.2011.09.018.

Klafke, G., Webster, A., Dall Agnol, B., Pradel, E., Silva, J., de La Canal, L.H., Becker, M., Osório, M.F., Mansson, M., Barreto, R., Scheffer, R., Souza, U.A., Corassini, V.B., dos Santos, J., Reck, J., Martins, J.R., 2017. Multiple resistance to acaricides in field populations of *Rhipicephalus microplus* from Rio Grande do Sul state, Southern Brazil. Ticks Tick. Borne. Dis. 8, 73–80. https://doi.org/10.1016/j.ttbdis.2016.09.019.

Lanusse, C., Lifschitz, A., Virkel, G., Alvarez, L., Sánchez, S., Sutra, J.F., Galtier, P., Alvinerie, M., 1997. Comparative plasma disposition kinetics of ivermectin, moxidectin and doramectin in cattle. J. Vet. Pharmacol. Ther. 20, 91–99. https://doi.org/10.1046/j.1365-2885.1997.00825.x.

LeOra Software, 2003. In: Robertson, J.L., Preisler, H.K., Russel, R.M. (Eds.), Polo Plus Probit and Logit Analysis, User's Guide. Berkeley, 36 pp.

Lifschitz, A., Virkel, G., Pis, A., Imperiale, F., Sanchez, S., Alvarez, L., Kujanek, R., Lanusse, C., 1999. Ivermectin disposition kinetics after subcutaneous and intramuscular administration of an oil-based formulation to cattle. Vet. Parasitol. 86, 203–215. https://doi.org/10.1016/S0304-4017(99)00142-9.

Lifschitz, A., Virkel, G., Sallovitz, J., Sutra, J.F., Galtier, P., Alvinerie, M., Lanusse, C., 2000. Comparative distribution of ivermectin and doramectin to parasite location tissues in cattle. Vet. Parasitol. 87, 327–338. https://doi.org/10.1016/S0304-4017(99)00175-2.

Lifschitz, A., Virkel, G., Ballent, M., Sallovitz, J., Imperiale, F., Pis, A., Lanusse, C., 2007. Ivermectin (3.15%) long-acting formulations in cattle: absorption pattern and pharmaco-kinetic considerations. Vet. Parasitol. 147, 303–310. https://doi.org/10.1016/j.vetpar.2007.04.009.

Lopes, W.D.Z., Teixeira, W.F.P., de Matos, L.V.S., Felippelli, G., Cruz, B.C., Maciel, W.G., Buzzulini, C., Fávero, F.C., Soares, V.E., Oliveira, G.P. de, da Costa, A.J., 2013. Effects of macrocyclic lactones on the reproductive parameters of engorged *Rhipicephalus (Boophilus) microplus* females detached from experimentally infested cattle. Exp. Parasitol. 135, 72–78. https://doi.org/10.1016/j.exppara.2013.06.003.

Nava, S., Rossner, M., Ballent, M., Mangold, A., Lanusse, C., Lifschitz, A., 2019. Relationship between pharmacokinetics of ivermectin (3.15%) and its efficacy to control the infestation with the tick *Rhipicephalus (Boophilus) microplus* in cattle. Vet. Parasitol. 268, 81–86. https://doi.org/10.1016/j.vetpar.2019.03.008.

Nava, S., Rossner, M.V., Torrents, J., Morel, N., Martinez, N.C., Mangold, A.J., Guglielmone, A., 2020. Management strategies to minimize the use of synthetic chemical acaricides in the control of the cattle tick *Rhipicephalus (Boophilus) microplus* (Canestrini, 1888) in an area highly favourable for its development in Argentina. Med. Vet. Entomol. 34, 264-278. https://doi.org/10.1111/mve.12432.

Reck, J., Marks, F.S., Rodrigues, R.O., Souza, U.A., Webster, A., Leite, R.C., Gonzales, J.C., Klafke, G.M., Martins, J.R., 2014. Does *Rhipicephalus microplus* tick infestation increase the risk for myiasis caused by *Cochliomyia hominivorax* in cattle? Prev. Vet. Med. 113, 59–62. https://doi.org/10.1016/j.prevetmed.2013.10.006.

Riviere, J.E., Sundlof, S.F., 2009. Chemical residues in tissues of food animals. In Veterinary Pharmacology and Therapeutics. 9th edn. Ed. Wiley-Blackwell, pp. 1453-1462.

Rodríguez-Vivas, R.I., Pérez-Cogollo, L.C., Rosado-Aguilar, J.A., Ojeda-Chi, M.M., Trinidad-Martinez, I., Miller, R.J., Li, A.Y., de León, A.P., Guerrero, F., Klafke, G., 2014. *Rhipicephalus (Boophilus) microplus* resistentes aos acaricidas e ivermectina nas fazendas de gado do México. Rev. Bras. Parasitol. Vet. 23, 113–122. https://doi.org/10.1590/S1984-29612014044.

Spath, E.J.A., Guglielmone, A.A., Signorini, A.R., Mangold, A.J., 1994. Estimación de las pérdidas económicas directas producidas por la garrapata *Boophilus microplus* y las enfermedades asociads en la Argentina. Therios. 23, 341–360.

Torrents, J., Sarli, M., Rossner, M. V, Toffaletti, J.R., Morel, N., Martínez, N.C., Webster, A., Mangold, A.J., Guglielmone, A.A., Nava, S., 2020. Resistance of the cattle tick *Rhipicephalus (Boophilus) microplus* to ivermectin in Argentina. Res. Vet. Sci. 132, 332–337. https://doi.org/10.1016/j.rvsc.2020.07.012.

Table 1. Prevalence (P), mean number, median (M) and first and third quartiles (1Q-3Q) of *Rhipicephalus (Boophilus) microplus* females 4.5–8.0 mm long of the treated (Group I; GI) and untreated (Group II; GII) groups of cattle. The therapeutic efficacy percentage (EP) is also shown. Cattle from Group I were treated with ivermectin 3.15% on days 0, 35 and 70.

	Group I			Group II				
Day post-treatment	P (%)	Mean number	M (1Q-3Q)	P (%)	Mean number	M (1Q-3Q)	EP (GI-GII)**	p-value*
14 (22 Jan 2020)	100	66	41(21-89)	100	97	94 (21-177)	NA	0.48
20 (28 Jan 2020)	90	13.2	7 (2-25)	100	54	65 (18-80)	75.6	0.01
35 (12 Feb 2020)	60	7.8	3 (0-11)	100	30	30 (11-49)	74.0	0.01
55 (03 Mar 2020)	10	0.2	0 (0-0)	75	20.5	11 (1-50)	99.0	0.009
70 (18 Mar 2020)	0	0	0 (0-0)	75	21.2	12 (0-47)	100	0.008
90 (07 Apr 2020)	0	0	0 (0-0)	100	56	62 (27-82)	100	0.0001
119 (04 May 2020)	0	0	0 (0-0)	100	92.4	96 (23-160)	100	0.0001
140 (27 May 2020)	10	0.2	0 (0-0)	100	50.4	38 (23-84)	99.6	0.001
170 (26 Jun 2020)	20	1	0 (0-1)	100	32.4	26 (17-51)	96.9	0.001
195 (21 Jul 2020)	30	1	0 (0-2)	100	41.6	42 (26-57)	97.6	0.001
202 (28 Jul 2020)	70	3.6	4 (0-7)	100	86.8	88 (59-115)	95.9	0.002

*Mann–Whitney test. Values are considered significantly different if P<0.01.

** The therapeutic efficacy percentage is presented only when the number of ticks in the treated group was significantly lower than the number of ticks in the control group.

NA: not applicable

Table 2. Results of the larval immersion test (LIT) with ivermectin applied to larvae of *Rhipicephalus (Boophilus) microplus* obtained from cattle after three successive treatments with ivermectin 3.15%.

Tick population	Slope ± S.E	LC_{50} (ppm)	CI95% LC_{50}	LC_{99} (ppm)	CI95% LC_{99}	RR_{50}	RR_{99}
I) Pre-treatment sample	11.74 ±1.44	8.73	7.6-9.1	13.2	11.9-15.34	-	-
II) Post-treatment sample (November 2020)	4.06 ± 0.20	10.5	9.7-11.2	39.2	33.2-48.6	1.2	2.9

LC: lethal concentration; ppm: parts per million; CI: confidence interval; RR_{50}: resistance ratio for LC_{50}; RR_{99}: resistance ratio for LC_{99}.

Figure captions:

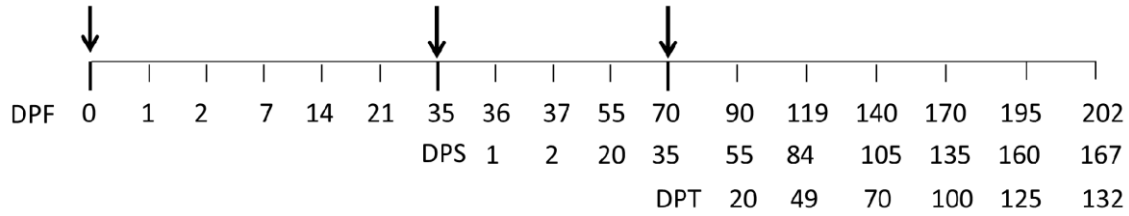

Fig. 1. Scheme of treatments with ivermectin 3.15%: i) arrows indicate the date of the application of each treatment; ii) numbers indicate the days post-treatments where counts of *Riphicephalus (Boophilus) microplus* females and take blood samples were performed. *R. microplus* counts began on day 14. DPF: days post-first treatment; DPS: days post-second treatment; DPT: days post-third treatment.

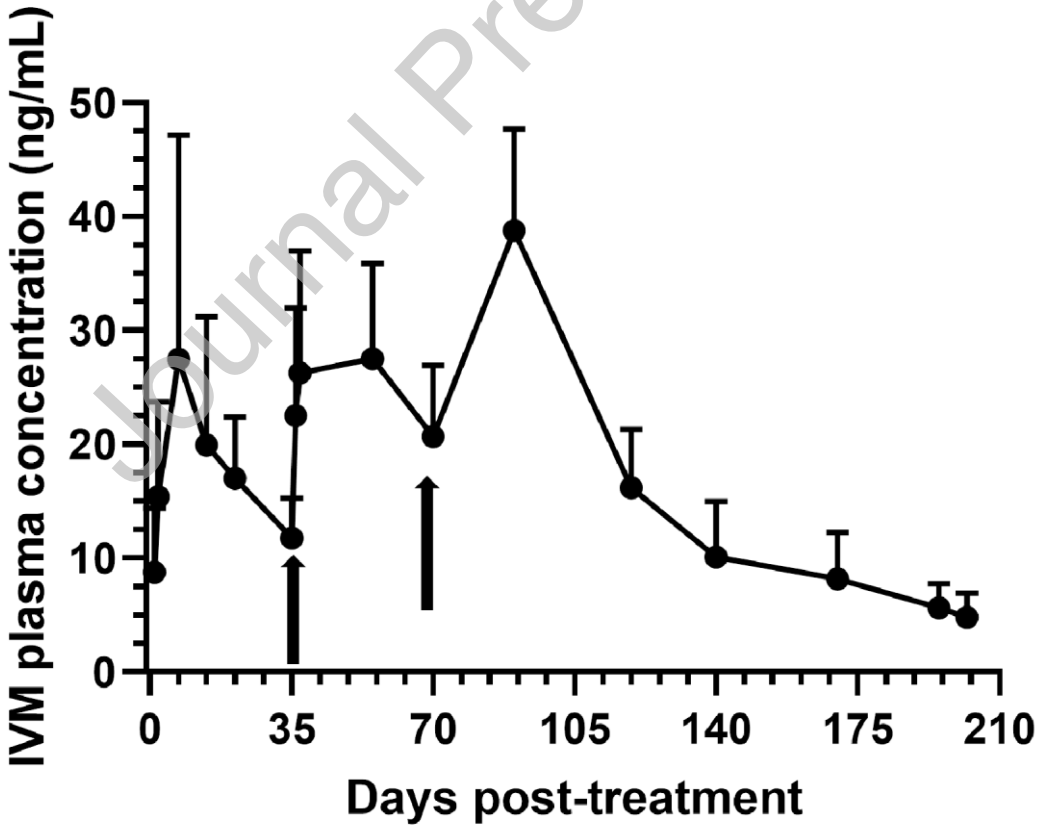

Fig. 2. Mean ivermectin (IVM) plasma concentrations (ng/mL) obtained after three successive treatments with a long-acting formulation (3.15%) at a dose rate of 630 µg/kg to cattle. The treatments were administered on days 0, 35 and 70. The arrows indicate the date of the second and third treatment.

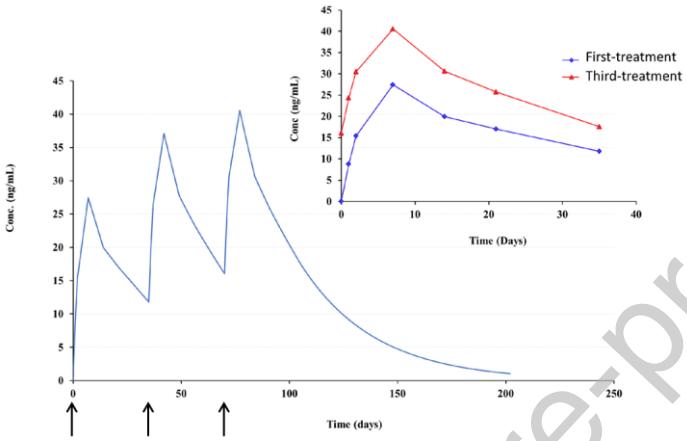

Fig. 3. Simulation of plasma ivermectin concentrations profiles obtained after three successive treatments with a long-acting formulation (3.15%) (days 0, 35 and 70). The simulation was made using the observed concentration profiles of ivermectin obtained after its administration on day 0. The insert shows the comparative concentration profiles observed after the first and third treatment. The arrows indicate the date of treatments.

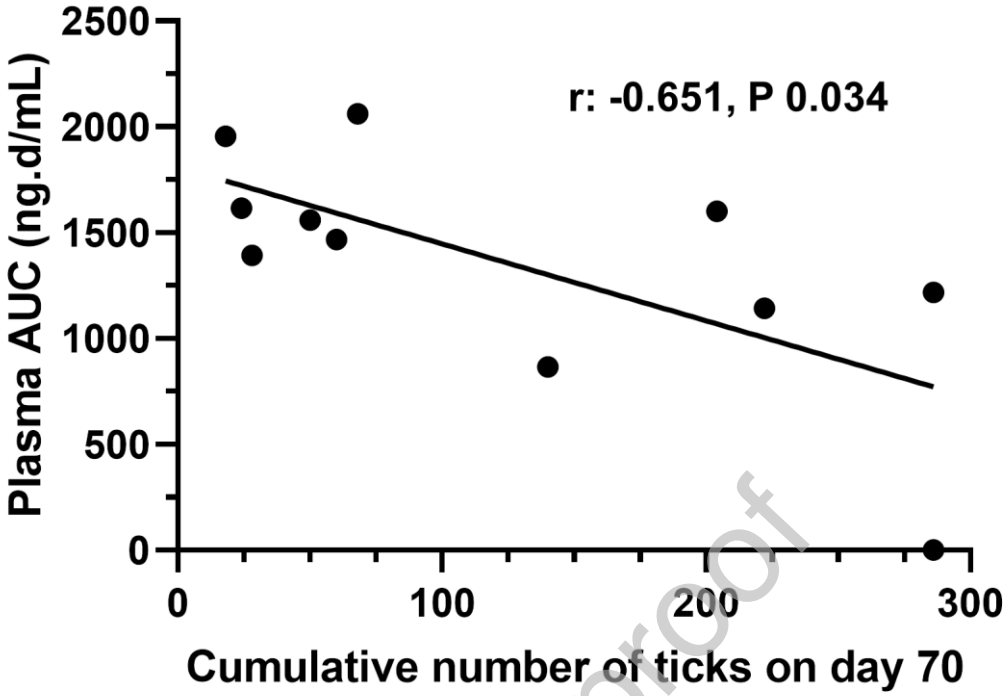

Fig. 4. Correlation between ivermectin systemic exposure measured as area under the concentration vs. time curves (AUC) and the cumulative number of ticks on day 70 post-first treatment.

Conflict of interest

The authors declare no conflicts of interest.