Weak phenotypic reversion of ivermectin resistance in a field resistant isolate of *Haemonchus contortus* by verapamil[1]

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ABSTRACT.- Borges F.A., Rossini J.B., Velludo P.P., Buzzulini C., Costa G.H., Molento M.B. & Costa A.J. 2011. **Weak phenotypic reversion of ivermectin resistance in a field resistant isolate of *Haemonchus contortus* by verapamil**. *Pesquisa Veterinária Brasileira 31(9):731-736*. Departamento de Medicina Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Campo Grande, MS 79070-900, Brazil. E-mail: fernando.borges@ ufms.br

Recent advances in anthelmintic resistant phenotype reversion by Pgp modulating drugs in ruminant nematodes indicate that this can be a useful tool to helminth control. The aim of the present study was to evaluate the efficacy of ivermectin (IVM) in combination with verapamil (VRP), in oil or water-based vehicle, against an IVM-resistant field isolate of *Haemonchus contortus* through a larval migration assay and experimental infection trial. In the *in vitro* assay was observed a phenotypic reversion of *H. contortus* resistance to ivermectin at a high concentration of VRP, increasing IVM efficacy from 53.1% to 94.3. In the *in vivo* trial, IVM + VRP demonstrated 36.02% efficacy compared to the 7.75% of IVM alone. The vehicle formulation showed no influence in efficacy. These are the first results demonstrating the effect of VRP as a partial IVM-resistance phenotype reverser in a field isolate of IVM-resistant *H. contortus* experimentally inoculated in sheep.

INDEX TERMS: *Haemonchus contortus*, ivermectin, P-glycoprotein, resistance, reversion, verapamil

RESUMO.- [**Reversão fenotípica da resistência a ivermectina em isolado de campo de *Haemonchus contortus* pelo verapamil.**] Avanços recentes na reversão fenotípica da resistência anti-helmíntica por drogas moduladoras de Pgp em nematódeos de ruminantes indicam que esta pode ser uma ferramenta útil no controle de helmintos. O objetivo do presente estudo foi avaliar a eficácia da ivermectina (IVM), em combinação com o verapamil (VRP), em veículo oleoso ou à base de água, contra um isolado de campo de *H. contortus* resistente por meio de teste de migração de larvas e infecção experimental em ovinos. No teste *in vitro*, observou-se reversão fenotípica da resistência de *Haemonchus contortus* à ivermectina com alta concentração de VRP, aumentando a eficácia da IVM de 53,1% para 94,3. No teste *in vivo*, IVM + VRP demonstrou 36,02% de eficácia em relação a 7,75% de IVM sozinha. O veículo da formulação não apresentou influência na eficácia. Estes são os primeiros resultados que demonstram o efeito da VRP como reversor parcial do fenótipo da resistência de IVM-fenótipo em um isolado de campo de *H. contortus* resistente, inoculado experimentalmente em ovinos.

TERMOS DE INDEXAÇÃO: *Haemonchus contortus*, ivermectin, P-glycoprotein, resistance, reversion, verapamil

INTRODUCTION

Macrocyclic lactones (ML) are a third-generation broad-spectrum anthelmintic agent for ruminants, but the exact resistance mechanisms are still far from being totally understood. Such mechanisms seem to be polygenic, involving several or many genes (Köhler 2001). Mutations in glutamate-gated chloride (GluCl) receptor genes are associated with ML-resistance (Blackhall et al. 1998), along with mutations in the dye-filling defective (*Dyf*) genes that encode proteins related to IVM uptake (Dent et al. 2000). The

first gene associated to ML resistance in *Haemonchus contortus* was Pgp-A, which encodes a P-glycoprotein, a member of the ATP-binding cassette transporter family (Xu et al. 1998). Pgp homologues are expressed in many organisms, including vertebrates, and are involved in the absorption, distribution, metabolism and excretion processes of xenobiotic compounds (Mealey 2004).

Pgp has been largely studied, as its overexpression can cause drug resistance in human tumor cells due to drug transport from the target cell, resulting in the phenomena known as multidrug resistance (MDR) when several chemical groups are involved (Juliano & Ling 1976, Gottesman & Pastan 1993). Pgp is also responsible for MDR in *Trypanosoma cruzi* and *T. brucei* brucei, which is a very important protozoan in human and animal health (Kerboeuf et al. 2003).

MDR reversion by modulating drugs is a recent treatment protocol employed in chemotherapy targeting tumor cells. These drugs directly act as competitive inhibitors of *Pgp*-mediated *drug* efflux and/or through indirect mechanisms that induce an increase in cellular ATP consumption and block the Pgp function (Watanabe et al. 1995, Garrigos et al. 1997). Verapamil (VRP) is a calcium channel blocker (Tsuruo et al. 1981) that has been used with this aim. There are a number of *in vitro* studies related to Pgp-activity modulation by VRP demonstrating partial reversion to benzimidazole and IVM resistance in nematodes (Beugnet et al. 1997, Molento & Prichard 1999, 2001, Bartley et al. 2009).

Along with the direct action of resistance reversion, VRP can significantly increase some pharmacokinetic parameters (AUC and Cmax) of *co-administrated* IVM in sheep, probably due to liver and intestinal Pgp inhibition, thereby reducing biliary excretion and increasing the intestinal absorption of IVM (Molento et al. 2004).

Lespine et al. (2008) pointed that the study of Pgp modulators should follow the following steps: selection of the drug, evaluation of the ability of this drug to modulate Pgp action in cells, pharmacokinetic studies in the host, *in vitro* tests with resistant parasites and *in vivo* coadministration of anthelmintic and Pgp modulator on experimentally infected hosts. These authors alerted to some possible problems such as side effects, different pharmacokinetic profile of the anthelmintic and the Pgp modulator, costs and the need of posterior studies on the withdraw period.

Recent studies showed the increase of ivermectin efficacy against resistant nematode in sheep (Lifschitz et al. 2010a) and cattle (Lifschitz et al. 2010b) experimentally infected and also the enhance of ivermectin systemic concentration by the coadministration of loperamide, a Pgp modulator.

All the studies on Pgp modulators in ruminants (Molento et al. 2004, Lifschitz et al. 2010a,b) used the drug diluted in saline solution. The use of a formulation containing the association of a Pgp modulator and ivermectin in an oil-based vehicle could reduce the rate of absorption from the injection site, resulting in prolonged residence time and longer effect of the drug on Pgp modulation.

Considering the absence of studies on Pgp-modulation by verapamil, a classical Pgp inhibitor, in sheep experimentally infected with an IVM-resistant field strain of *Haemonchus contortus*, the aim of the present study was to evaluate the *in vitro* and *in vivo* efficacy of ivermectin co-administered with verapamil and the possible effect of the vehicle formulation.

MATERIALS AND METHODS

The Ivermectin-resistant field strain of *Haemonchus contortus* was isolated from the Ovine Sector of the School of Agrarian and Veterinary Sciences, São Paulo State University, Jaboticabal, São Paulo, Brazil. A previous controlled anthelmintic efficacy test following the methodology recommended by Wood et al. (1995) confirmed the extremely high phenotypic resistance status of this field strain of *H. contortus* to IVM, that was ineffective (-33.97% efficacy).

For the isolation of the monospecific strain, *H. contortus* females were obtained from abomasums and immediately transferred to a saline solution in Petri dishes at 36ºC for two hours. The eggs produced were transferred to recipients containing vermiculite and incubated at 27ºC for 10 days, when the third-stage infective larvae were obtained. Two worm-free sheep were inoculated with 1000 viable L3 larvae and maintained in individual pens at the Animal Health Research Center (CPPAR/Unesp) in order to minimize accidental re-infection.

Infective larvae from an IVM-resistant field strain of *H. contortus* were used in the *in vitro* agar migration assay test (D'Assonville et al. 1996, modified by Molento & Prichard, 2001). The larvae were submitted to the following treatments: Group I: 1, 2, 4, 8, 16, 32, 64, 128 and 256µM of IVM (ivermectin, Ivomec® Solução Oral, Merial Saúde Animal); Group II: 1, 2, 4, 8, 16, 32, 64, 128 and 256 µM of IVM co-administered with 2mM of VRP (verapamil hydrochloride, Sigma, St Luis, Missouri, USA) - this concentration of VRP showed to be able to increase the efficacy of IVM in other work (Molento & Prichard 2001); Group III: 1, 2, 5, 10 and 100mM of VRP; Group IV: IVM EC$_{50}$ co-administered with 0, 1, 2, 5, 10 and 100mM of VRP.

The drugs were diluted in distilled water, instead of oil to avoid interference in the larvae migration in agar, and vortexed immediately prior to use. In each experimental group, there was a triplicate that only received distilled water, used as the control group. The larva number used in each of the four groups was variable, depending on the availability. Thus, efficacy percentages between treatments were statistically compared rather than mean larva survival rate.

The *in vivo* evaluation of reversion of ivermectin resistance design and procedures were performed based on the guidelines of the WAAVP (Wood et al. 1995) and VICH (Vercruysse et al. 2001). The management of the experimental sheep was carried out in compliance with the Ethics Committee of the institution and "Good Clinical Practice" guidelines (VICH GL9, 2000, http://vich.eudra.org/pdf/2000/GL09_st7.pdf).

As it was not possible to produce a large number of naive animals, the decision was made to use lambs that were treated with an anthelmintic agent until the total elimination of any pre-experimental nematode infection. All animals were housed in stalls designed to avoid parasitic infections and provided with daily food and water *ad libitum*.

After an acclimation period, each worm-free animal was orally inoculated with 8460 IVM-resistant *H. contortus* L3 larvae. Thirty of the 35 inoculated sheep were selected based on eggs per gram (EPG) counts on Days 32, 33 and 34 post-inoculation. The animals were ranked based on the EPG count and allocated into five groups (n=6) in a randomized block design: I- Control; II- 1% IVM (200 µg/kg); III- 15% VRP (3mg/kg), water-based vehicle, administrated three times at 12-hour intervals; IV- 1% IVM (200 µg/kg), single dose, co-administered with 15% VRP (3mg/kg), water-based vehicle, administrated three times at 12-hour intervals (separate formulations); V- 1% IVM (200µg /kg) plus 15% VRP (3mg/kg),

oil-based vehicle (sterile pure corn oil*)*, single dose (combined formulation).

All treatments were performed subcutaneously on Day 35 post-inoculation and the animals were observed for possible adverse reactions. The established VRP dose of 3mg/kg is the safest for sheep when diluted in saline solution and administered subcutaneously (Molento et al. 2004). The aim of using an oil-based vehicle is to allow slow absorption of the drug from the injection site, as VRP is absorbed and eliminated very quickly, whereas macrocyclic lactones have a longer absorption half-life and mean residence time.

All sheep were sacrificed seven days post-treatment and subjected to a parasitological necropsy in order to determine the worm burden. Fecal samples were collected and eggs per gram (EPG) were determined for each animal (Gordon & Whitlock 1939) in order to evaluate the effect of the treatments on nematode reproduction.

The half maximal effective concentration (EC_{50}) of IVM against the resistant field strain was calculated using the GraphPad Prism software program (GraphPad Prism, version 5.01 for Windows, San Diego, California, USA, www.graphpad.com). The total number of surviving larvae in each treatment was log-transformed and normalized to fit a dose-response sigmoid curve. The curves were statistically compared (fitted values log EC50 and Hill slope) by the Tukey's multiple comparison test. The total *H. contortus* number recovered at the necropsy of each animal was transformed in log x+1. The therapeutic efficacy of the formulations was calculated based on the method described by Wood et al. (1995). The fecal egg count reduction (FECR) was calculated based on the guidelines of the US Food and Agriculture Organization (FAO 2004): FECR % = [1- (*C1/C2*) × (*T2/T1*)] × 100, in which T and C are the geometric means for the treated and control groups and subscripts 1 and 2 designate the counts before and after treatment, respectively. In the controlled anthelmintic test, the D'Agostino-Pearson normality test was used to quantify how far the distribution is from Gaussian in terms of asymmetry and shape. The data were transformed and one-way analysis of variance (ANOVA) with Tukey's Multiple Comparison *post hoc* test was performed on the log-transformed data.

RESULTS

The *in vitro* test results of IVM alone and associated to VRP are displayed in Figure 1 and 2. Table 1 displays the total number of surviving larvae exposed to VRP alone and its association with EC_{50} IVM as well as the efficacy of the drugs.

The IVM sigmoid dose-response curve was not statistically significantly (P>0,05) different from IVM associated to VRP (2mM) (Fig.1). The EC_{50} for IVM alone was 4.314μM, with a 95% confidence interval ranging from 3.045 to 5.900 μM,
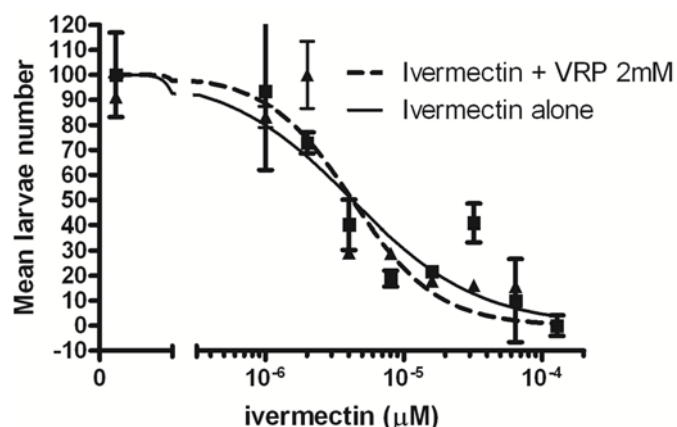

Fig.1. *In vitro* agar migration assay test results. Dose-response sigmoid curve of IVM alone and IVM associated with VRP (2 mM) against IVM-resistant field isolate of *Haemonchus contortus* (normalized-transformed data)
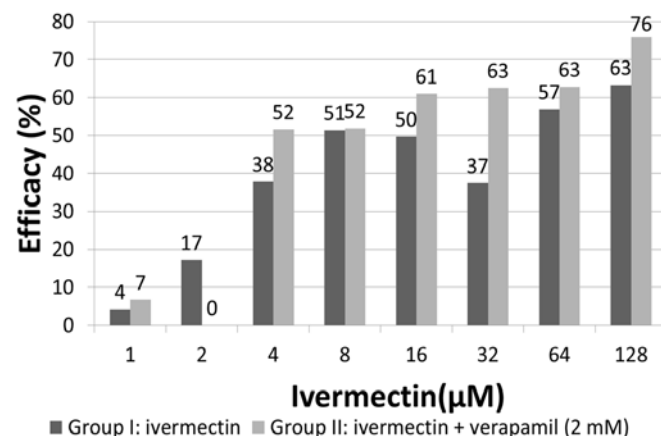

Fig.2. *In vitro* agar migration assay test results. Efficacy (%) of IVM alone and IVM associated with VRP (2 mM) against IVM-resistant field isolate of *Haemonchus contortus*

compared to 3.045ìM for IVM plus VRP (2mM), ranging from 3.047 to 5.898μM (95% confidence interval).

The presence of VRP (2mM) did not reduced (P=0.074) the number of surviving larvae, although it was observed greater efficacy of IVM associated with VRP (Fig.2), except at the dilution of 2μM/0.5mL (Fig.2). The efficacy of 128ìM of IVM plus 2mM of VRP was 12.55% higher than 128μM of IVM alone.

Table1. *In vitro* agar migration assay test results, mean number of surviving *Haemonchus contortus* larvae, and efficacy of VRP alone (Group III) and associated with IVM EC_{50} (Group IV)

	Group III: verapamil			Group IV: Ivermectin (EC_{50} 4.314 μM) + verapamil			Adjusted efficacy(%)
Concentration (mM)	Mean number of larvae(SE[b])	Efficacy (%)	Verapamil concentration(mM)	Mean number of larvae(SE[b])	Efficacy (%)		
zero[a]	467[a] (14.52)	-	zero	219[ab] (6.48)	53.1		53.1
1	420[a] (5.77)	10.0	1	310[a] (1.66)	33.6		23.6
2	417[a] (24.03)	10.7	2	177[c] (35.11)	62.1		51.4
5	417[a] (21.85)	10.7	5	157[c] (6.66)	66.4		53.7
10	430[a] (11.54)	7.9	10	180[bc] (14.01)	61.4		53.5
100	458[a] (14.33)	1.9	100	27[d] (15.19)	94.3		92.4

* Control triplicates = larvae were treated with distilled water alone. **SE = Standard error. ***Adjusted efficacy = efficacy of group IV - efficacy of group III. [a,b,c,d] = mean values in the same column followed by at least one common letter do not differ by Tukey's multiple comparison test (P>0,05).

Table 2. *In vivo* results. Geometric means of EPG counts and number of adult *Haemonchus contortus* recovered at necropsy

Treatment		EPG		H. contortus		
		D zero	D+7	Male	Female	Total
Control	Mean	3520	7240	451	546	1016
verapamil	Mean	3533	11576	480	597	1087
3mg/kg(aq.)	Efficacy	-	0.00%	0.00%	0.00%	0.00%
ivermectin	Mean	3533	8166	408	506	937
200μg/kg	Efficacy	-	0.00%	9.43%	7.26%	7.75%
ivermectin 200μg/kg +	Mean	3415	10006	316	316	650
verapamil 3mg/kg (aq.)	Efficacy	-	0.00%	29.89%	42.15%	36.02%
ivermectin 200μg/kg +	Mean	3585	6165	415	566	995
verapamil 3mg/kg (oil)	Efficacy	-	16.39%	7.97%	0.00%	2.14%
One-way ANOVA; Significant			0.86			0.94
P-value (P<0.05)			No			No

VRP administrated alone had no action against nematode parasites (Table 1), even at high concentrations (100mM). However, increasing concentrations of VRP associated to IVM EC_{50} resulted in an increase in IVM efficacy against the resistant *H. contortus* isolate (Table 1). IVM (EC_{50}) associated to 2, 5 or 100mM of VRP caused a significant reduction in the number of larvae (P<0.05) when compared to IVM EC_{50} alone. IVM (EC_{50}) associated to 100 mM of VRP showed 94.3% efficacy against the resistant *H.contortus* isolate, while IVM (EC50) alone caused the mortality of 53.1% of the larvae, as expected. To exclude possible direct verapamil effect on *Haemonchus contortus*, it was calculated the adjusted efficacy (efficacy of IVM plus VRP - efficacy of VRP alone) shown on Table 1.

No local or systemic adverse reactions were observed in the sheep following VRP administration. The *in vitro* results revealed that VRP administered alone, at 1, 2, 5, 10 and 100mM, had no anthelmintic effect.

The FECR results (Table 2) revealed an increase in the number of *H. contortus* eggs eliminated in the feces of all animals in comparison to Day 0 (prior to treatment). The 1% IVM + 15% VRP (oil-based vehicle) treatment demonstrated limited efficacy (16.39%) in the reduction of EPG, but the mean egg number was not statistically different from the control group (P>0.05). The other treatments had no efficacy with regard to FECR.

The controlled anthelmintic test results demonstrated no efficacy of the IVM, thereby confirming the resistant phenotype status of this *H. contortus* isolate (Table 2). The FECR and the controlled test results were similar, except for 1% IVM (200 μg/kg), single dose, co-administered with 15% VRP (3mg/kg) (water-based vehicle), administrated three times at 12-hour intervals (separate formulations), which demonstrated a general efficacy of 36.02% (29.89% against *H. contortus* males and 42.15% against females). This value was 4.65-fold higher than IVM alone (7.75%), thereby demonstrating a partial reversion of resistance. One percent IVM (200μg /kg) plus 15% VRP (3mg/kg) (oil-based vehicle) in a combined formulation had no efficacy against this resistant *H. contortus* isolate, as there were a high number of post-treatment parasites.

DISCUSSION

The *in vitro* agar gel larva migration assay was a practical, inexpensive, fast, quantitative method for assessing anthel-mintic efficacy and could be an important tool for determining anthelmintic resistance in nematodes found in ruminants. Using the same methodology, but a laboratory-selected isolate, Molento & Prichard (2001) observed different results to the present work. Fifty-five percent efficacy was found for IVM alone and 84% for IVM associated with 2mM of VRP. In the present study, this concentration of verapamil was not suffi-cient to increase the efficacy of IVM and only high VRP levels (100mM) could increase IVM from 53.1% to 94.3%. It is an interesting result, since the same methodology, nematode specie and drug concentrations were used, remarkable diffe-rences in efficacy were observed.

Partial reversion of the resistance of *Haemonchus contortus* to another chemical group (benzimidazole) occurred in an egg hatch test involving both resistant and susceptible isolates, for which VRP caused an increase in efficacy of thiabendazole and albendazole EC_{50}, with more visible action in resistant rather than susceptible isolates (Beugnet et al. 1997). Other *in vitro* study demonstrated the action of a different Pgp modulator. Prior exposure of *H. contortus* eggs to lectin resulted in a thiabendazole EC_{50} reduction of 50.9% in a susceptible isolate and 47.2% and only 27.4% against two resistant isolates.

There are only a few studies that have tested the effect of Pgp modulators for increasing the anthelmintic efficacy in animal hosts infected by *H. contortus*. In one experiment conducted in Brazil, VRP associated to IVM was evaluated in naturally infected sheep and 74.71% efficacy was observed, while IVM alone demonstrated no efficacy (Borges et al. 2005). Molento & Prichard (1999) used an experimental model with jirds (*Meriones unguiculatus*) infected with an IVM-resistant *H. contortus* isolate and found 26% efficacy with IVM alone and 48% when associated with VRP. In the same experimen-tal model, Xu et al. (1998) used a *H. contortus* isolate selected with moxidectin and observed 80% and 70% efficacy of IVM and moxidectin when administrated alone and 90% and 96% efficacy when associated to VRP, respectively. The VRP dose was much higher in the latter experiment (20mg/kg) than in the present work (3mg/kg).

The first anthelmintic controlled tests with experimentally infected ruminants were carried out by Lifschitz et al. (2010a) in sheep and Lifschitz et al. (2010b) in cattle. Loperamide increased the efficacy of IVM from 23 to 50% and of moxidectin from 69 to 87% against a resistant *Cooperia*

oncophora isolate in cattle and the efficacy of IVM from 0% to 72.5% against *H. contortus* in sheep.

These results are different from the observed in the present study. Co-administered VRP and IVM in a combined formulation with oil-based vehicle caused no increase in IVM anthelmintic efficacy against the IVM-resistant *H. contortus* isolate, as there were a large number of parasites in the animals with this treatment. These results show the influence of the formulation over anthelmintic efficacy, as the same compounds in water-based vehicle achieved satisfactory results. It was expected that the oil vehicle formulation would show higher efficacy because it would allow a slow absorption of VRP and consequently more long-acting modulation of Pgp. until IVM could achieve the peak plasma concentration. Future studies on the pharmacokinetics of the VRP and IVM formulation in sheep will help answer questions regarding the vehicle and drug concentrations.

It was observed a remarkable difference between the FECRT and the anthelmintic controlled test, especially in the treatment IVM (200 ìg/kg) plus 15% VRP (3 mg/kg) in water-based vehicle. In FECRT, this group showed 0% efficacy while in the anthelmintic controlled test it was observed efficacy of 36.02%. According to Coles et al. (2006), the anthelmintic controlled test controlled efficacy test is the most reliable method and is considered the gold standard for detecting anthelmintic resistance. FECRT can be affected by the fecundity of the female worms due to density-related modulation of egg output by female, as described by Koop e t al. (2008) *in Ancylostoma caninum*. these authors observed that egg counts in the treated dogs were 41% higher 6 days after treatment, despite the killing of 71% of the adult worms.

Pouliot et al. (1997) evaluated the hydrophobicity of ivermectin and VRP and MDR reversion and found ivermectin to be nine-fold more hydrophobic than VRP; consequently, ivermectin was found to be a better inhibitor of drug transport by Pgp. The structure/affinity relationship for the interaction between Pgp and macrocyclic lactones (ivermectin, abamectin, eprinomectin, doramectin, selamectin and moxidectin) and the Pgp inhibitors valspodar and VRP was evaluated by Lespine et al. (2007), who found a considerable variation in the transport rate of the drugs by Pgp. IVM was a 10-fold and 7.23-fold more potent substrate of Pgp than moxidectin and VRP, respectively. These results show that IVM has greater affinity and is extensively more transported by Pgp than VRP. Thus, it is expected that VRP would not be the best Pgp modulator for IVM-resistance reversion in *H. contortus* as demonstrated by Bartley et al. (2009) that used a cell culture model test and observed that there are significant differences between the concentrations of ivermectin, ketoconazole, quercetin, pluronic P85, valspodar and verapamil to inhibit the Pgp transport activity. In addition, verapamil showed to be a better Pgp modulator than ketoconazole but a least potent Pgp inhibitor than valspodar, ivermectin and pluronic P85 in a larval feeding inhibition test with resistant isolates of *Teladorsagia circumcincta* and *H. contortus*.

Although verapamil showed to be a potent Pgp modulator in previous studies (Beugnet et al. 1997, Molento & Prichard 1999, Bartley et al. 2009) it was not efficient in reverting the phenotype in the *H. contortus* isolate evaluated in the present

work. These are the first results demonstrating the effect of VRP as a partial IVM-resistance phenotype reverser in a field isolate of IVM-resistant *H. contortus* experimentally inoculated in sheep. The study of the physiochemical properties of Pgp drug modulators may reveal molecules with a greater binding and transport rate than IVM, resulting in better Pgp inhibition and, consequently, higher anthelmintic-resistance phenotype reversion than that observed for VRP in the present experiment and by other authors.

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