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RESISTÊNCIA ANTI-HELMÍNTICA DE NEMATÓDEOS GASTROINTESTINAIS DE RUMINANTES NATURALMENTE INFECTADOS NO ESTADO DO RIO GRANDE DO SUL, BRASIL

Santa Maria, RS
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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Mestre em Medicina Veterinária.**

Orientador: Profª Dr.ª Fernanda Silveira Flores Vogel

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Aprovado em 19 de fevereiro, 2016:

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Santa Maria, RS
2016

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RESUMO

RESISTÊNCIA ANTI-HELMÍNTICA DE NEMATÓDEOS GASTROINTESTINAIS DE RUMINANTES NATURALMENTE INFECTADOS NO ESTADO DO RIO GRANDE DO SUL, BRASIL

AUTOR: Fernanda Ramos
ORIENTADOR: Fernanda Silveira Flores Vogel

Entre as principais enfermidades que afetam ruminantes ao redor do mundo, as infecções por nematódeos do trato gastrointestinal ocupam papel de destaque. Estas infecções impactam negativamente o desempenho dos animais, comprometendo a viabilidade econômica dos mesmos, uma vez que não se faça o controle adequado destes parasitas. Neste sentido, foi realizada a avaliação de diferentes compostos com ação anti-helmíntica em bovinos e ovinos no estado do Rio Grande do Sul. O primeiro capítulo, apresenta o estudo cujo objetivo foi avaliar a resistência anti-helmíntica de nematódeos gastrointestinais de bovinos naturalmente infectados à diferentes anti-helmínticos comercialmente disponíveis no estado do Rio Grande do Sul; e testar a eficácia de combinações destes princípios sobre populações multirresistentes. Para tanto, foram selecionados 70 a 100 bovinos, com idades entre sete a nove meses, naturalmente infectados e com contagens de ovos por grama de fezes (OPG) ≥200. Estes indivíduos eram oriundos de 10 propriedades diferentes localizadas no Rio Grande do Sul, as quais não haviam realizado tratamento anti-helmíntico nos 60 dias precedentes ao início do estudo. Os animais foram aleatoriamente alocados em dez grupos (7- 10 animais cada) e tratados com ivermectina, doramectina, eprinomectina, fenbendazole, closantel, nitroxinil, disofenol, levamisole, albendazole ou moxidectina. Grupos adicionais de 7 a 10 bezerros foram utilizados para testar seis combinações, de duas drogas cada, em quatro das propriedades estudadas. De maneira geral o fenbendazole foi a droga mais eficaz, seguido pelo levamisole, disofenol e moxidectina. Parasitas dos gêneros *Cooperia*, *Trichostrongylus* e *Haemonchus* foram os mais resistentes aos diferentes tratamentos empregados. As combinações de moxidectina+levamisole, doramectina+fenbendazole e levamisole+closantel foram as mais eficazes. Os resultados deste estudo demonstraram a existência de populações multirresistentes a maioria dos anti-helmínticos comercialmente disponíveis, enquanto combinações destes podem representar uma alternativa para melhora de eficácia dos tratamentos. O segundo capítulo teve por objetivo verificar a eficácia do monepantel (Zolvix ®) sobre nematódeos gastrointestinais de cordeiros naturalmente infectados oriundos de duas propriedades da região central do estado do Rio Grande do Sul, Brasil. Para tanto, 64 animais sendo, 17 animais tratados com zolvix e 17 animais controle não tratados na propriedade 1, e 20 animais tratados com o mesmo princípio e 10 controle na propriedade 2, foram utilizados. Em ambas as propriedades, os animais não receberam tratamento anti-helmíntico nos 30 dias anteriores ao estudo e possuíam OPG ≥200. A eficácia do monepantel foi de 25,8% e 78,4%, respectivamente, nas propriedades 1 e 2, sendo que os gêneros que apresentaram resistência foram *Haemonchus*, *Trichostrongylus* e *Cooperia*. Demonstrou-se que a resistência anti-helmíntica em ovinos vem se agravando dada a ineficácia da última droga lançada no mercado brasileiro em combater a infecção dos animais, sendo necessários mais estudos para retardar o aparecimento de novos casos de resistência a este e aos demais compostos disponíveis comercialmente.

Palavras-chave: Bovinos. Ovinos. Teste de Eficácia. Helmintos Gastrointestinais.

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ABSTRACT

ANTHELMINTHIC RESISTANCE OF GASTROINTESTINAL NEMATODES OF RUMINANTS NATURALLY INFECTED IN THE STATE OF RIO GRANDE DO SUL, BRAZIL

AUTHOR: Fernanda Ramos
ADVISER: Fernanda Silveira Flores Vogel

Among the main diseases that affect ruminants around the world, infections by gastrointestinal nematodes display an important role. These infections, negatively impact the performance of the animals, compromising the economic viability of the systems, if the correct control of these parasites is not made. In this sense, were performed the evaluation of different compounds with anthelminthic action in sheep and cattle in the state of Rio Grande do Sul. The first chapter presents the study whose objective was to evaluate the anthelminthic resistance of gastrointestinal nematodes from naturally infected cattle with different anti-helminthic therapy commercially available in the state of Rio Grande do Sul; and test the effectiveness of combinations of these principles on multiresistant populations. For both, were selected 70-100 animals, with ages between 7 to 9 months, naturally infected and with egg counts per gram of feces (EPG) ≥200. These individuals were from ten different property located in Rio Grande do Sul, which had received no anthelmintic treatment in the 60 days preceding the beginning of the study. The animals were randomly divided in ten groups (7- 10 animals each) and treated with ivermectin, doramectin, eprinomectin, fenbendazole, closantel, disophenol, nitroxynil, albendazole, levamisole or moxidectin. Additional groups of 7 to 10 calves were used to test six combinations of two drugs each in four of studied properties. In general, the fenbendazole was the most effective drug, followed by levamisole, disofenol and moxidectin. However parasites of genera *Cooperia*, *Trichostrongylus* and *Haemonchus* were the most resistant to different treatments employed. The combinations of moxidectin+levamisole, doramectin+fenbendazole and levamisole+closantel were the most effective. The results of this study demonstrated the existence of multidrug-resistant populations to most of the anthelmintic substances commercially available, while combinations of these may represent an alternative for improvement of the efficacy of treatments. The second chapter had as objective to verify the efficacy of monepantel (Zolvix ®) on gastrointestinal nematodes of naturally infected lambs from two properties in the central region of the state of Rio Grande do Sul, Brazil. For it, 64 animals being, 17 animals treated with Zolvix and 17 control animals not treated in property 1, and 20 animals treated with the same principle and 10 control on the property 2, were used. In both properties, the animals did not receive any anthelmintic treatment in the 30 days prior to the study and had EPG ≥200. The efficacy of monepantel was 25.8% and 78.4%, respectively, in the properties 1 and 2, being *Haemonchus* spp., *Trichostrongylus* spp. and *Cooperia* spp. the genera that presented greater resistance to it. It was demonstrated that the status of anthelminthic resistance in sheep is getting worse once the last drug launched in the Brazilian market for the treatment of gastrointestinal infections of the animals proved ineffective, and further studies are needed to delay the appearance of new cases of resistance to this drug and the other compounds commercially available.

Keywords: Cattle. Sheep. Efficacy Test. Gastrointestinal Helminths

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1 INTRODUÇÃO

A produção de bovinos e ovinos têm, historicamente, grande destaque dentre as principais cadeias produtivas do setor de carnes no Brasil. Atualmente, o país conta com o segundo maior rebanho comercial de bovinos do mundo e é o décimo oitavo maior produtor de ovinos, contando com 17.6 milhões de cabeças desta espécie, distribuídas em diferentes regiões do seu território (IBGE, 2014). A pecuária brasileira se caracteriza por ter a maior parte do rebanho criado a pasto, sendo a área de pastejo estimada em 174 milhões de hectares (LOBATO et al., 2014). Desta forma, o país é detentor de uma das formas mais econômicas e práticas de oferecer alimentos para os ruminantes, gerando menos custos de produção destes animais em comparação a outros sistemas praticados em diversas regiões do mundo (DIAS-FILHO, 2010; FERRAZ e FELÍCIO, 2010).

Dentre os desafios inerentes a criação de ruminantes estão às infecções por endoparasitas, as quais determinam muitos prejuízos econômicos aos produtores por afetarem o bem-estar e desenvolvimento dos animais. Particularmente, o parasitismo por nematódeos do trato gastrointestinal, constitui um dos principais entraves na criação de ruminantes, comprometendo o desempenho reprodutivo, o sistema imunológico, a produção de leite e de carne, além de reduzir o ganho de peso e a conversão alimentar. Contudo, na maioria dos casos, as endoparasitoses gastrointestinais, cursam de forma subclínica, e suas consequências muitas vezes passam despercebidas pelos produtores (WEST et al., 2009; COSTA et al., 2004; SOUZA et al., 2008). Em virtude disso, para que se mantenha o desempenho dos rebanhos de forma geral, é essencial que se apliquem medidas corretas e eficazes de controle e profilaxia, visando minimizar os efeitos dessas parasitoses, mantendo-as em níveis aceitáveis, compatíveis com a intensidade do sistema de produção dos animais (MOLENTO et al., 2009)

O surgimento de drogas com amplo espectro de ação, grande eficácia e poder residual, incentivou o uso destes medicamentos pelos proprietários por sua facilidade de adaptação aos diferentes sistemas de criação, substituindo a atividade dos médicos veterinários como consultores em sanidade animal e, até mesmo, do próprio diagnóstico das parasitoses gastrointestinais (FAO, 2003). Práticas desta natureza, atreladas a facilidade de acesso aos produtos anti-helmínticos e do uso seu extensivo, comprometem a manutenção da refugia e a eficácia dos tratamentos, conforme pode ser observado pela dimensão dos problemas de

resistência parasitária disseminados nas mais diferentes regiões do mundo (TAYLOR; HUNT; GOODYEAR, 2002; SOUTELLO et al, 2010; DEMELER et al., 2009).

A refugia compreende o grupo de larvas e ovos que permanecem na pastagem sem sofrer ação das drogas, consistindo em um estoque de larvas susceptíveis (COSTA, SIMÕES E RIET-CORREA, 2011). Da mesma forma, parasitas adultos ou estágios imaturos albergados em animais não tratados, atuam como uma população em refugia, apresentando um papel fundamental na sustentabilidade dos tratamentos antiparasitários (van WYK et al., 2006; MOLENTO, 2009). Isso se deve ao fato de que quanto maior a parcela da população parasitária exposta a doses, terapêuticas ou não, dos antiparasitários utilizados no rebanho, maior será a pressão de seleção ou o estímulo para a sobrevivência e proliferação de genótipos parasitários com maior resistência aos princípios ativos empregados. Assim, a manutenção da refugia contribui para a diluição dos genes que codificam para resistência anti-helmíntica nas próximas gerações, retardando o processo de seleção (WOLSTENHOLME et al., 2004; KENYON et al. 2009).

Neste sentido, uma vez que se realizem rotações de bases químicas de forma criteriosa, a pressão de seleção para determinada base em detrimento de outra é interrompida, eliminando-se muitos genótipos resistentes à primeira, pela utilização da segunda, dificultando, assim, o desenvolvimento da resistência (JACKSON e COOP, 2000). Em contrapartida, a utilização equivocada dos principais anti-helmínticos disponíveis comercialmente, através da alternância incorreta de bases químicas e alta frequência de tratamentos, aliada a transferência de cepas resistentes de um rebanho à outro pela aquisição de animais, resultaram no aparecimento de diversos casos de resistência parasitária múltipla, lateral e, possivelmente, cruzada (RAJAN et al. 2002; SILVESTRE et al., 2000; LEATHWICK; POMROY; HEATH, 2001; LEATHWICK et al., 2009).

O desenvolvimento da resistência dos parasitas gastrointestinais aos anti-helmínticos se dá de forma gradual e seu reconhecimento em estágios iniciais só é possível por meio do monitoramento da eficácia dos tratamentos (McKELLAR e JACKSON, 2004). Os métodos para detecção de resistência parasitária compreendem testes *in vivo* e *in vitro*, porém, a disponibilidade de testes *in vitro* validados para o diagnóstico da resistência ainda é limitada, além de haverem poucos laboratórios que ofereçam este tipo de serviço (FORTES & MOLENTO, 2013). Por outro lado, dos testes *in vivo* disponíveis, o teste de redução da contagem de ovos nas fezes é amplamente difundido na literatura e de fácil execução, podendo ser utilizado para todos os grupos de anti-helmínticos (COLES et al., 2006).

Desta forma, fica evidente que o controle das infecções parasitárias por helmintos é essencial para o sucesso dos sistemas de produção de ruminantes e deve se basear em um bom conhecimento da epidemiologia básica, das particularidades regionais, técnicas de manejo e tipo de sistema produtivo (CEZAR; CATTO e BIANCHIN, 2008). Além disso, o desenvolvimento e aplicação de métodos que auxiliem a detectar e monitorar o processo de desenvolvimento da resistência parasitária são imprescindíveis para a manutenção da viabilidade econômica dos sistemas de criação (TAYLOR; HUNT; GOODYEAR, 2002).

Os prejuízos associados a tratamentos anti-helmínticos ineficazes tendem a ser maiores quando tratamos de sistemas intensivos de criação, seja pela aplicação incorreta dos diferentes compostos ou pela resistência parasitária (TOMAZ-SOCCOL et al. 2004). Assim, a ineficácia de diferentes antiparasitários frente aos nematódeos gastrointestinais foi descrita primeiramente em pequenos ruminantes, principalmente nos ovinos (FARIAS et al, 1997; CEZAR et al., 2010a) em consequência à maior intensidade de tratamentos com anti-helmínticos e uso de fármacos de longa ação no rebanho (CEZAR et al., 2011). Da mesma forma, esta problemática vem sendo amplamente descrita em bovinos (SOUTELLO et al, 2010; SOUZA et al, 2008), inclusive em diversas regiões do Brasil, como nos estados de Minas Gerais (RANGEL et al. 2005), São Paulo (SOUTELLO; SENO; AMARANTE, 2007), Mato Grosso do Sul (BORGES et al. 2013) e Santa Catarina (SOUZA et al. 2008).

No Rio Grande do Sul alguns estudos, como os de Echevarria et al. (1996), Mello et al. (2006) e Cezar et al. (2010b) demonstram que a resistência anti-helmíntica a princípios ativos do grupo das avermectinas/milbemicinas, benzimidazóis e imidazotiazóis é um problema presente na criação de bovinos e ovinos em algumas regiões do estado, sendo que para estes últimos, em algumas propriedades, restam poucas alternativas farmacológicas conforme demonstrado pelos resultados de Cezar et al. (2010a). Por conseguinte, a coleta de dados que demonstrem a real dimensão desta problemática no estado, tanto em grandes como pequenos ruminantes, é eminente.

Tendo em vista o grande impacto econômico das parasitoses gastrointestinais nos animais de produção atrelado à baixa expectativa de surgimento de novas drogas antiparasitárias, nos capítulos seguintes serão apresentados e discutidos experimentos com a finalidade de apresentarem dados atuais sobre a resistência parasitária em bovinos e ovinos oriundos de diferentes rebanhos do estado do Rio Grande do Sul, os quais se propuseram, mais especificamente, ao objetivo de: i)avaliar a resistência de parasitos gastrointestinais de bovinos de corte naturalmente infectados a diferentes princípios com ação anti-helmíntica comercialmente disponíveis no Rio Grande do Sul; ii) testar a eficácia de combinações destes

fármacos como uma alternativa sobre populações de nematódeos gastrointestinais multirresistentes; iii) verificar a eficácia do monepantel (Zolvix ®) contra nematódeos gastrointestinais de ovinos naturalmente infectados oriundos de duas propriedades da região central do estado do Rio Grande do Sul, Brasil. Esta dissertação está dividida em dois capítulos, sendo o primeiro intitulado "Anthelmintic resistance in gastrointestinal nematodes of beef cattle in the state of Rio Grande do Sul, Brazil" e o segundo "Resistência anti-helmíntica de nematódeos gastrointestinais de ovinos ao monepantel na região central do estado do Rio Grande do Sul, Brasil".

2 ARTIGO 1

(Artigo submetido à revista "International Journal for Parasitology: drugs and drug resistance")

Anthelmintic resistance in gastrointestinal nematodes of beef cattle in the state of Rio Grande do Sul, Brazil

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Abstract**.** Gastrointestinal nematodes resistant to anthelmintics have been reported in several regions of Brazil, and they may be associated with economic losses for the cattle industry. This study aimed to evaluate the resistance status of gastrointestinal nematodes from naturally infected beef cattle to several commercially available anthelmintics, as well as to test the efficacy of combinations of anthelmintics against multi-resistant gastrointestinal nematodes. Ten farms located in Rio Grande do Sul state were selected by: farmers' consent; extensive raising system; availability of calves aged from 7 to 9 months naturally infected by gastrointestinal nematodes; absence of anthelmintic treatment for 60 days before the study; and presence of 70 to 100 calves or more of both genders with ≥200 eggs per gram of feces (EPG) (sensitivity of 50 EPG). These calves were distributed into 10 groups (of 7 to 10 animals) per farm and treated with ivermectin, doramectin, eprinomectin, fenbendazole, closantel, nitroxynil, disophenol, levamisole, albendazole, or moxidectin. Feces were collected 2 days before treatment and 14 days after treatment. Additional groups of 7–10 calves were used to test six different two-drug combinations at four of the studied farms. In general terms, fenbendazole was the most effective drug, followed by levamisole, disophenol, and moxidectin. However, parasite resistance to multiple drugs was found in all herds, especially in the genera *Cooperia* spp., *Trichostrongylus* spp., and *Haemonchus* spp.. Some of the two-drug combinations were effective against nematode populations identified as resistant to the same compounds when used as single drugs. The most effective combinations were moxidectin + levamisole, doramectin + fenbendazole, and levamisole + closantel. In this study, parasites resistant to the main commercially available anthelmintics were found in all herds, and some combinations of two active components belonging to different chemical groups were effective against multi-drug resistant gastrointestinal nematodes.

Keywords: endoparasites, FECRT, bovine, multidrug resistance.

1. Introduction

The cattle industry is one of the largest sectors of the Brazilian economy. Brazil is the world's second largest producer of cattle, with a total herd of 217.4 million head (FAO, 2014; Cider, 2014). Recently, the cattle industry has experienced a rise in intensity and productivity, as shown by a 50% increase in occupancy rate (animal/hectare) and a 3.4% decrease in pasture area from 1990 to 2011 (INSTITUTO FNP, 2012). Particularly in the state of Rio Grande do Sul, beef cattle production occurs predominantly on native pastures, often without considering the effects on sustainability (Beretta et al., 2002) and the environmental changes caused by increased population density and restriction of livestock movement. In addition, genetic selection for desired production characteristics has led to changes in the natural parasite/host balance, resulting in increased susceptibility of cattle to parasites (Waller, 2002).

Infections by gastrointestinal nematodes affect the well-being and productivity of hosts, causing decreased reproductive performance, a low growth rate, weight loss, and poor food conversion (Mello et al., 2006; West et al., 2009; De Graef et al., 2013). In Brazil, anthelmintics are generally used at farmers' discretion, with no restrictions to access to commercially available drugs and without any assistance from veterinarians. Thus, inadequate use of anthelmintics is not rare; indeed, animals are often treated excessively, interfering with production, accelerating selection of resistant parasites, and posing significant problems for the cattle industry (Delgado et al., 2009; Zanetti Lopes et al., 2013).

Parasite resistance has gradually become a significant problem facing cattle producers in several regions worldwide, including Brazil (de Souza et al., 2008; Demeler et al., 2009). Limited information exists regarding parasite resistance status in local cattle herds in the Brazilian state of Rio Grande do Sul; however, there is strong evidence that gastrointestinal nematodes infecting Brazilian herds have gained resistance to the main available classes of anthelmintics (Soutello et al., 2007, Cezar et al., 2010b; Borges et al., 2013; Neves et al., 2014).

This study aimed to verify the existence of populations of gastrointestinal nematodes resistant to several commercially available anthelminthic compounds by evaluating naturally infected beef cattle from herds located in the state of Rio Grande do Sul, Brazil. In addition, the efficacies of some two-drug combinations were tested to assess their potential as alternative to control the multi-drug resistant parasite populations found in the studied herds.

2. Material and methods

2.1 Farms and animals

The study was conducted on ten farms located in eight counties of the Rio Grande do Sul state in southern Brazil: São Martinho da Serra, Dilermando de Aguiar (two farms), Cacequi (two farms), São Gabriel, Itaqui, São Borja, Santiago, and São Vicente do Sul (Figure 1). Preliminarily, herds were selected based on location and previous consent by farmers. Additionally, the following technical criteria were considered: the extensive system used to raise beef cattle; the availability of *Bos taurus*/*Bos indicus* crossbred calves of both genders (aging from 7 to 9 months); the presence of 70 to 100 calves or more per farm with counts of ≥200 eggs per gram of feces (EPG); and the absence of anthelmintic treatment for 60 days before the experimental period. First, all calves available at each farm were included in the study; however, animals with fewer than 200 EPG before treatment were excluded prior to the formation of the experimental groups. Calves were weaned approximately six months after birth and kept in the same grazing area before and during the study on each farm. The use of animals was approved by the Committee of Ethics in Animal Experimentation of the Federal University of Santa Maria under protocol no. 3132240215.

2.2 Anthelmintic treatment

In the first part of the study, ten commercially available anthelmintic compounds were tested on each farm. All treatments were administered by a veterinarian participant of the study following the manufacturer's recommendations: ivermectin 1% (0.2 mg/kg, subcutaneous, Hipramectin® HIPRA), doramectin 1% (0.2 mg/kg, subcutaneous, Dectomax® Zoetis), eprinomectin 0.5% (500 µg/kg, pour-on, Eprinex® Merial), moxidectin 1% (0.2 mg/kg, subcutaneous, Cydectin® Ford

Dodge), levamisole 7.5% (3.75 mg/kg, subcutaneous, Ripercol L® Fort Dodge), albendazole 15% (3.4 mg/kg, subcutaneous, Agebendazol® Agener), nitroxynil 34% (9.7 mg/kg, subcutaneous, Dovenix Supra®, Merial), disophenol 20% (5 mg/kg, subcutaneous, Pradoverme® PRADO), fenbendazole 10% (5 mg/kg, oral, Panacur® Intervet), and closantel 10% (10 mg/kg, oral, Diantel® HIPRA).

After determining the efficacy of each single anthelmintic treatment, six combinations of two drugs were tested at four of the ten farms as a second part of this study. For this purpose, new groups of calves, selected by the criteria described before, were used. The drug combinations were based on the results of the first part of this study and selected according to the recommendations of Cezar et al. (2011), Geary et al. (2012), and Pivoto et al. (2014). The choice of two-drug combinations was made with a focus on including different modes of action and efficacy against different genera of gastrointestinal nematodes. The tested combinations were: moxidectin 1% (0.2 mg/kg, subcutaneous, Cydectin® Ford Dodge) + levamisole 7.5% (3.75 mg/kg, subcutaneous, Ripercol L® Fort Dodge), moxidectin 1% (0.2 mg/kg, subcutaneous, Cydectin® Ford Dodge) + albendazole 15% (3.4 mg/kg, subcutaneous, Agebendazol® Gener), albendazole 15% (3.4 mg/kg, subcutaneous, Agebendazol® Agener) + closantel 10% (10 mg/kg, oral, Diantel® HIPRA), doramectin 1% (0.2 mg/kg, subcutaneous, Dectomax® Zoetis) + closantel 10% (10 mg/kg, oral, Diantel® HIPRA), doramectin 1% (0.2 mg/kg, subcutaneous , Dectomax® Zoetis) + fenbendazole 10% (5 mg/kg, oral, Panacur® Intervet), and levamisole 7.5% (3.75 mg/kg, subcutaneous, Ripercol L® Fort Dodge) + closantel 10% (10 mg/kg, oral, Diantel® HIPRA). Each drug in the combination treatments was administered separately.

2.3 Experimental groups and fecal analysis

Samples were collected directly from the rectum of each calf 2 days prior to treatment (D−2) and on day 14 after treatment (D+14) according to the recommendations of Coles et al. (2006). All samples were collected in plastic bags, labeled, stored in isothermal boxes for transport to the laboratory, maintained at 10 °C for up to 12 hours after collection, and processed, as recommended

by McKenna (1998). All samples were maintained under controlled humidity and temperature before processing and during the larvae culture procedures.

Counting of EPG was performed by a McMaster modified technique, with a sensitivity of 50 EPG. Briefly, each sample of 4 g of homogenized feces was mixed and diluted in 56 mL of saturated solution, re-suspended, sifted, and transferred to a McMaster chamber for EPG counting by microscopic identification. Animals that had an EPG count ≥200 on D-2 were selected. These calves were distributed into 10 randomized blocks based on EPG at each farm, to balance the mean and the frequency distributions of EPG countings among the groups before the treatments. Each of the ten groups was randomly treated with a single drug in the first part of this study. At the four farms included in the second part of this study, six additional groups were treated with a combination of two anthelmintic compounds as described previously. The number of animals in each experimental group ranged from 7 to 10 depending on the available calves at each farm. The total number of calves used per farm was: 257 (farm 1), 110 (farm 2), 205 (farm 3), 108 (farm 4), 127 (farm 5), 138 (farm 6), 264 (farm 7), 184 (farm 8), 181 (farm 9), 130 (farm 10).

On each collection day, fecal samples from all calves in each experimental group were pooled, mixed with sterile wood shavings, and stored for larvae cultures (moisturized daily with sterile water under incubation for seven days at 22–27 °C and 80% humidity), according to the recommendations of Coles et al. (2006). After incubation, larvae were recovered by baermanization, after which 100 third-stage larvae in each culture were identified (by genera) following the criteria described by Van Wyk and Mayhew (2013).

2.4 Statistical analysis

On each farm, pre-treatment and post-treatment EPG counts were used to calculate the efficacy of each treatment based on the reduction in EPG. For this purpose, the approach described by Torgerson et al. (2014) was used (available at http://www.math.uzh.ch/as/index.php?id=254&L=1). The selected approach incorporated random sampling error and aggregations between individual hosts in the treatment groups to provide 95%

confidence intervals, which were taken as the 2.5 and 97.5 percentiles of the resulting efficacy distribution.

The efficacy of each treatment against each genus of gastrointestinal nematodes was calculated based on the proportion of each genus of nematode in the larvae cultures at D-2 and D+14 using the following formula: $PR = 100 \times (1 - PER_{final}/PER_{initial})$, where *PR* is the percentage reduction by genus; and *PERinitial* and *PERfinal* are the percentages of each genus before (D-2) and 14 days after (D+14) treatment, respectively (Coles et al., 1992; Coles et al., 2006; Neves et al., 2014).

2.5 Interpretation of the results

Anthelmintic resistance status was interpreted as recommended by Lyndal-Murphy et al. (2014) and based on the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines on anthelmintic resistance (Coles et al., 1992), considering the EPG reduction percentage and the upper (UCL) and lower (LCL) 95% confidence limits. Therefore, each treatment was classified as effective (when the EPG reduction percentage and upper 95% confidence limit were both equal or above 95% and the lower 95% confidence limit was equal or above 90%), ineffective (parasite resistance confirmed, when the EPG reduction percentage and upper 95% confidence limit were below 95% and the lower 95% confidence limit was below 90%), or inconclusive (when none of the other criteria were fulfilled). Moreover, multi-drug resistant parasites were defined as parasite populations of gastrointestinal nematodes that were resistant to anthelmintic drugs of different chemical classes according to the recommendations of James et al. (2009).

3. Results

Arithmetic means, minimum EPG counts, maximum EPG counts, and the percentages of each genus of gastrointestinal nematodes found before treatment in each herd are shown in Table 1. Table 2 presents the efficacy of each treatment at each farm. Table 3 shows the percentage reduction of each genus after each treatment at each farm. The presence of gastrointestinal nematodes with resistance to multiple anthelmintic compounds was detected in all evaluated herds;

on 60% (6/10) of the farms, nine of the ten active compounds tested had efficacy <90% (Table 2). Fenbendazole was the most effective compound in the studied herds, followed by levamisole, disophenol, and moxidectin. Larvae cultures from animals from all herds showed the presence of mixed infections containing the following genera: *Haemonchus*, *Cooperia*, *Oesophagostomum*, *Trichostrongylus*, and *Ostertagia* (Table 1). *Oesophagostomum* spp. were the most susceptible of the identified genera to anthelmintic compounds, whereas *Cooperia* spp. were the most resistant, followed by *Trichostrongylus* spp. and *Haemonchus* spp. (Table 4).

Treatment of the animals with avermectin compounds did not result in satisfactory EPG reduction in any herd. Moxidectin was fully effective at one farm, but unsatisfying reductions in EPG counts were observed in the other nine herds. With regard to the benzimidazoles employed in this study, albendazole was ineffective against gastrointestinal parasites at nine farms and showed an inconclusive result at the farm 10. Fenbendazole was effective at farms 2 and 10 and resulted in lower, but not negligible, EPG reductions of approximately 90% at farms 1, 5, 7, 8, and 9. In the same way, levamisole exhibited outstanding efficacy greater than 95% at farms 9 and 10; however, levamisole produced no similar reduction in EPG count at the other six tested farms.

Considering the narrow spectrum compounds tested, closantel had unsatisfying results at 90% (9/10) of the farms. However, closantel was effective against *Haemonchus* spp. at farms 1, 5, 6, 8, and 10, while it showed no action against *Cooperia* spp. Phenolic-substituted compounds disophenol and nitroxynil showed differing efficacy. Nitroxynil was ineffective at all farms when EPG reduction was considered, mainly because it had little effect on *Cooperia* spp., but it was effective against *Haemonchus* spp. on farms 1, 6, 8, and 9. Disophenol was not effective at six farms, showed inconclusive results at three farms (1, 6, and 10), and had an efficacy of 96.3% at farm 9. Disophenol was effective against *Haemonchus* spp. at farm 1 and *Ostertagia* spp. at farms 1, 4, 5, and 7. The efficacy of each two-drug combination is presented in Table 5. Some combinations were highly effective, surpassing 95% efficacy. The most effective treatment was moxidectin 1% + levamisole 7.5%, followed by doramectin 1% + fenbendazole 10%, which presented some inconclusive results

with efficacy of approximately 90%. Table 6 shows the effect of each anthelmintic combination on each genus of gastrointestinal nematodes at each farm. Table 7 shows the mean efficacy of each anthelmintic combination against gastrointestinal nematode genera found at all farms. In general, the same genera identified as resistant to single drugs were found to be resistant to two-drug combinations. However, some groups showed large reductions in EPG counts after treatment with anthelmintic combinations, resulting in a lack of viable larvae after treatment (D+14) (Table 6 and Table 7).

4. Discussion

Resistance of gastrointestinal nematodes infecting cattle to some classes of anthelmintic compounds has been demonstrated in Brazilian herds in the states of Santa Catarina, São Paulo, and Mato Grosso do Sul by Souza et al. (2008), Condi et al. (2009), and Almeida et al. (2013), respectively. However, the results of the present study indicate a worrying situation in relation to the control of gastrointestinal nematodes infections in cattle herds from Rio Grande do Sul because of the high level of multi-drug resistance of the parasite populations found in all farms studied. The broad detection of parasite resistance to several anthelmintics recognized as good quality commercial drugs suggests that parasite populations have developed resistance to the main classes of anthelmintic drugs available in Brazil.

Macrocyclic lactones (MLs), especially avermectins, were not effective in any of the herds assessed in this study, with the exception of moxidectin at one farm. Similar results were found in other cattle herds by Mello et al. (2006), Cezar et al. (2010b), and Lagunes et al. (2015). Mello et al. (2006) and Demeler et al. (2009) reported that MLs are the most commonly used class of compounds for the control of gastrointestinal helminths in ruminants because of their broad spectrum and endectocide activity, which encourage excessive use and have led to resistance. In the farms evaluated here, no detailed information was obtained regarding the history of each drug at each farm because of a lack of available data. Drug use on Brazilian farms is often not based on established criteria, while trademarks and compound names are not well recognized by the farmers.

As an exception, ivermectin is well recognized and the most widely used anthelmintic, followed by other avermectins, benzimidazoles, levamisole, and cydectin. Other compounds are eventually used when the farmer suspects that conventional drugs are failing. Commercial availability, endectocide action, and price are generally considered most important criteria influencing the choice of drugs by farmers.

Proportionally to the other genera of gastrointestinal nematodes found in the tested herds, *Cooperia* spp. larvae showed lower susceptibility to MLs (Table 3 and Table 4). Resistance of *Cooperia* spp. to MLs is not rare; however, treatment failure is often not perceived by farmers because of the low pathogenicity of some species of *Cooperia* (except, for example, *C. oncophora* and *C. punctata*) (Cezar et al., 2010b; Fazzio et al., 2014; Zanetti Lopes et al., 2014). Nevertheless, massive infections by *Cooperia* spp. can lead to loss of appetite, diarrhea, and decreased weight gain (Demeler et al. 2009). Despite the presence of resistant populations of *Cooperia* spp. in the studied herds, clinical signs were not apparent in calves. Moreover, larvae of the genera *Trichostrongylus*, *Haemonchus*, *Ostertagia*, and *Oesophagostomum* were identified as resistant after treatment with MLs; however, these genera were not present in samples from all farms (Table 3).

Levamisole, an imidazothiazole derivative, was a good alternative for the treatment of gastrointestinal nematodes at some farms, in line with reports by Duarte et al. (2012) and Gasbarre (2014). While farmers reported knowledge of levamisole in the present study, it was not frequently used, indicating low selection pressure. This condition may have contributed to the good efficacy of levamisole at some farms. A similar result was found by Molento et al. (2013) regarding sheep in Brazil, where reintroduction of levamisole in a flock that had not been exposed to it for 10 years resulted in efficacy of more than 95%. However, in the present study, with the exception of *Oesophagostomum* spp., other genera were not fully controlled by levamisole, corroborating the data obtained by de Souza et al. (2008) and Neves et al. (2014).

Phenolic substitutes nitroxynil and disophenol are narrow spectrum anthelmintics that are not recommended in the presence of infections by *Cooperia* spp., *Trichostrongylus* spp., or

Ostertagia spp.; however, they are indicated to control *Haemonchus* spp., which is associated with a decrease in food consumption, weight loss, and loss of productivity in cattle (Mckellar and Jackson, 2004; Gasbarre, 2014). Nitroxynil and Disophenol were ineffective in reducing the EPG in most herds, mainly because of the presence of genera of gastrointestinal nematodes that were not sensitive to these compounds. Some strains of *Oesophagostomum* spp. and *Ostertagia* spp. were susceptible to nitroxynil and disophenol; however, resistance of *Haemonchus* spp. to nitroxynil and disophenol was detected in some herds. Nitroxynil was effective against *Haemonchus* spp. at farms 1, 6, 8, and 9, whereas disophenol was effective against *Haemonchus* spp. at farm 1 and *Ostertagia* spp. at farms 1, 4, 5, and 7. These results show that phenolic-substituted drugs have limited applicability in the studied cattle herds.

Benzimidazoles (BZs), including albendazole and fenbendazole, are broad-spectrum drugs widely used as anthelmintics in ruminants worldwide (De Graef et al., 2013). Yazwinski et al. (2009), Cezar et al. (2010b), and Demeler et al. (2008) reported efficacies >95% for these anthelmintics in large ruminants. However, in the present study, albendazole had efficacy <90% at all tested farms, while fenbendazole was highly effective at only 2 farms. Frequent use of BZs at the studied farms may have resulted in the establishment of benzimidazole-resistant parasite populations. Considering the location of the farms, these data suggest that parasite resistance to BZs may be spreading in Rio Grande do Sul, similar to the situation observed for avermectins in several Brazilian herds. The resistance of *Cooperia* spp., *Haemonchus spp.*, and *Trichostrongylus* spp. to BZs at most farms was similar to the results reported by Yazwinski et al. (2009).

Closantel presents a narrow spectrum of action against gastrointestinal nematodes of ruminants. In Brazil, closantel (Diantel®) is recommended mainly to control *Haemonchus* spp. infections in sheep and cattle. Thus, closantel can be considered as a treatment for controlling gastrointestinal nematodes in certain conditions (Costa et al., 1996). Although closantel was not previously used in any of the studied cattle herds, it did not control infection by gastrointestinal nematodes at 90% of the tested farms. *Cooperia* spp. (the least sensitive genus), *Trichostrongylus*

spp., and *Ostertagia* spp. were not susceptible to closantel in most cases. Closantel was effective against *Haemonchus* spp. at farms 1, 5, 6, 8, and 10, but *Haemonchus* spp. were resistant to closantel on farms 3, 4, 7, and 9. While this is the first report of gastrointestinal nematode resistance to closantel in cattle herds of the state of Rio Grande do Sul, resistance to this compound has been reported by Costa et al. (1986 and 1996) in the state of São Paulo. Furthermore, closantel resistance is very common in sheep, as reported at several studies, due to its intensive use on small ruminants (Cezar et al., 2010a; Sczesny-Moraes et al., 2010; Verissimo et al., 2012).

Multi-drug resistance occurs when multiple classes of anthelmintics no longer control certain parasitic populations that originally consisted of a large majority (more than 95%) of susceptible genotypes (Taylor et al., 2009). Multi-drug resistance is very common among the main types of gastrointestinal nematodes that infect sheep and goats; indeed, multi-drug resistance is an emerging issue in cattle around the world, including those raised in Brazil and a number of European countries (Rangel et al., 2005; Geurden et al., 2015). The low efficacy of each single drug and the presence of multi-drug resistant gastrointestinal nematodes infecting cattle are major problems that prevent adequate anthelmintic control at the farms evaluated in this study. Thus, more sustainable strategies of anthelmintic control in ruminants are required to overcome the problem of multi-drug resistance (Cezar et al., 2011; Geary et al., 2012).

Given that the main classes of anthelmintics did not reduce the EPG of treated calves, combinations of active compounds were administered as an alternative treatment approach (Bartram et al., 2012). Similar to the results of a study performed by Cezar et al. (2011) in sheep, two-drug combinations of anthelmintics were tested on cattle herds in the present work based on previous tests of the efficacy of single drugs. Therefore, previous knowledge regarding parasite resistance was used as a tool to inform the choice of potentially efficacious combinations of drugs. The use of combinations of two anthelmintic compounds with good efficacy as single drugs could be an effective means of delaying the development of drug resistance in parasites. However, this study was focused on situations in which two effective drugs were unavailable to farmers. Thus,

combinations of two anthelmintics that were not fully effective as single drugs, had different modes of action, had broad spectra of action (when possible), and were effective against different genera of gastrointestinal nematodes were tested.

Some of the anthelmintic combinations were effective against multi-drug-resistant parasite populations, reaching EPG reduction percentages ≥95% (Table 5 and Table 6). Despite the unsatisfying efficacies of moxidectin and levamisole as single drugs, the combination of moxidectin 1% + levamisole 7.5% was effective in all four evaluated herds. The combination of doramectin 1% + fenbendazole 10% was highly effective at farm 8. Acceptable efficacy was shown by some combinations: moxidectin 1% + albendazole 15% at farms 3 and 8, doramectin 1% + fenbendazole 10% at farm 3, and levamisole 7.5% + closantel 10% at farm 1. The success of this practice can be justified by the fact that combination of drugs belonging to unrelated chemical groups, with different mechanisms of action, impairs the survival of the parasite genotypes adapted to one of the single compounds (Geerts and Gryseels, 2000; Hu et al., 2010). Many of the tested combinations were not effective, probably because of the presence of genotypes of gastrointestinal nematodes resistant to both drugs used in the combinations.

The results of this study showed the presence of gastrointestinal nematodes resistant to the main commercially available anthelmintic drugs on cattle farms evaluated in the state of Rio Grande do Sul, Brazil. In critical situations of parasite resistance, in which no options of effective drugs are commercially available, combinations of two anthelmintic compounds with different mechanisms of action and unsatisfying efficacy as single drugs can effectively control multi-drug resistant gastrointestinal nematodes. However, such combinations should be evaluated under the particular conditions unique to each farm at which cattle are raised.

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Figure 1. Location of ten beef cattle herds studied at eight counties from the state of Rio Grande do Sul in southern Brazil. The black spheres indicate the locations of the farms.

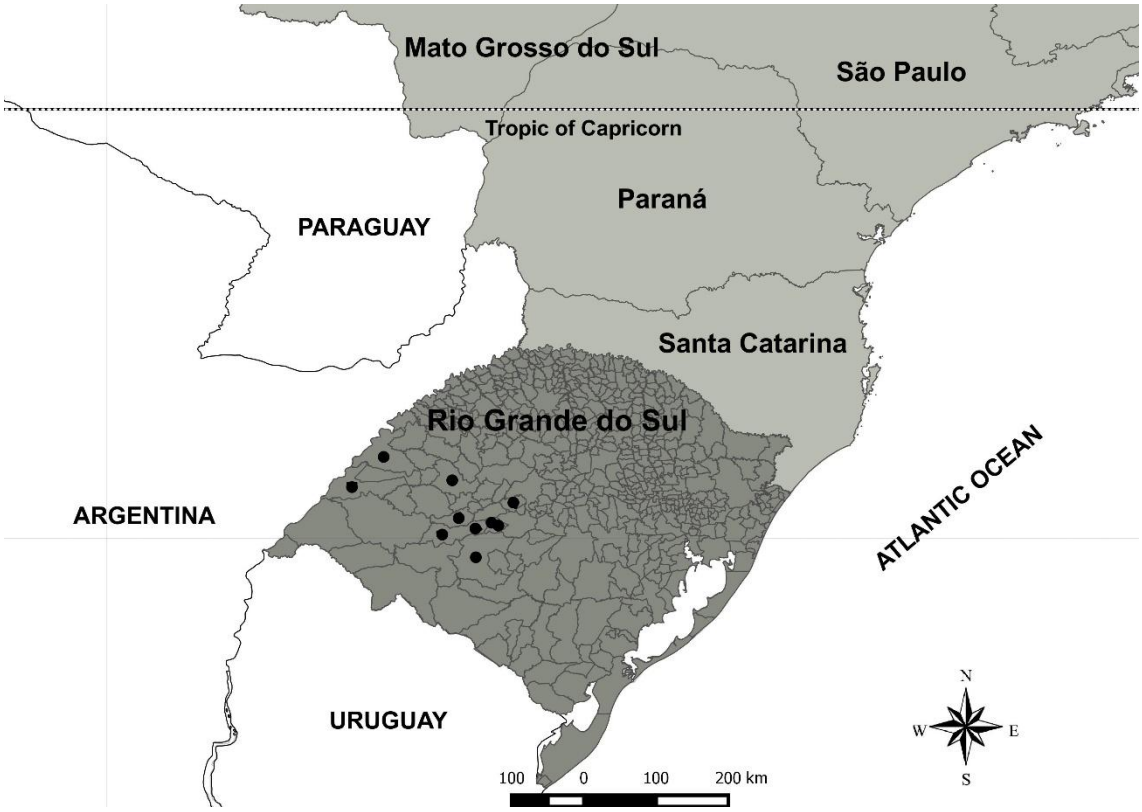

Table 1. Arithmetic mean (AM) and standard deviation (SD), minimum (MIN) and maximum (MAX) fecal egg counts and percentage of genera identified before the treatments (D-2) in the feces of naturally infected beef cattle from ten farms in the state of Rio Grande do Sul.

Farms	EPG			Genera of the gastrointestinal nematodes (%) .				
	AM (SD)	MIN	MAX	*Cooperia* spp.	*Oesophagostomum* spp.	*Haemonchus* spp.	*Ostertagia* spp.	*Trichostrongylus* spp.
1	440.85 (±346.2)	200	1550	70	26	4	0	0
2	249.1 (±156.5)	200	2700	78	22	0	0	0
3	623.5 (±501.6)	200	2850	60	8	0	4	28
4	413.6 (±225.9)	200	1250	76	0	16	2	6
5	368.5 (±226.3)	200	1200	20	0	40	10	30
6	776.2 (±593.6)	200	3600	34	0	10	0	56
7	1657.3 (±1223.2)	200	6850	40	6	44	2	8
8	806.8 (±605.3)	200	2550	87	7	6	0	0
9	624.4 (±467.7)	200	2350	68	14	8	0	0
10	476.7 (±296.1)	200	1350	64	10	26	0	0

Table 2. Percentage of EPG reduction (and 95% confidence interval) calculated by the fecal egg count reduction test (FECRT) fourteen days after anthelmintic treatment in beef cattle naturally infected by gastrointestinal nematodes on ten farms in the state of Rio Grande do Sul, Brazil.

Anthelmintic treatments	Reduction of EPG after treatment on each farm									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Ivermectin 1%	11.6 (-28.1-39.5)	17.6 (-13 – 39.2)	-99.1 (-138- -61.4)	21.8 (-12.8- 42.9)	56.6 (33.8-72.8)	17.4 (-1.87 – 33.7)	69.6 (62.7-74.4)	45.2 (28.1-59)	-99 (-143 - -63.8)	61.8 (44.6-76.2)
Doramectin 1%	-55.6 (-55.4–23.8)	-28.4 (-64 -47.2)	-46 (-82.5 - -20)	80.9 (67.2-90)	29.7 (0.67 – 52.5)	-36.9 (-74.1- -11.2)	26.9 (15.2-34.4)	-1.82 (-25-23.4)	-21.6 (-47.2 - -4.21)	42.6 (13.2-62.8)
Eprinomectin 0.5%	67.8 (46.4-80.7)	81.2 (70.4-88.6)	-9.92 (-45 -14.8)	50.5 (21.8-68.8)	33.1 (-5.8- 53.3)	13.6 (-7.5 – 28)	63.8 (56.6-69.7)	21.3 (-2.8 -38.7)	12.4 (-6.6 - 32.1)	69.3 (54.5-80.7)
Moxidectin 1%	63.7 (41.2 - 77.7)	76.7 (64.8-85.8)	65.1 (52.5-75.9)	78.7 (65.3-88.7)	83.7 (71.3-92)	80.1 (71.5-86.7)	90.4 (87.2-93.2)	78 (66.1-85.6)	64.4 (49.2-74.9)	99.2 (95.1-100)
Albendazole 15%	13.4 (-20.3 – 37.7)	60.2 (44.1-71.9)	-28.2 (-61.2-15.2)	29.1 (0.51 – 51.9)	-4.3 (-39.5- 27.2)	37.9 (20.4-50.5)	57.4 (49.9-63.8)	79.3 (70.3-86.1)	29.3 (10.1-44.7)	87 (74.1-94.3)
Levamisole 7.5%	81.9 (65.3 - 91.3)	84.7 (74.9-91.5)	70.6 (52-82.3)	90.2 (77.6-96.6)	34.8 (0.8 – 55.9)	46.1 (31-58.4)	71.7 (64.6-77.5)	93 (87.4-96.9)	97.5 (94.2-99.2)	97.7 (91.8- 99.7)
Nitroxynil 34%	71.7 (50.4 - 84.2)	48.3 (22-64.8)	-13.4 (-40.3 – 10.5)	86.1 (73-93.7)	23.2 (-11.8-48)	31.1 (12.6-45.9)	72.8 (65.6-78.5)	51.6 (37.4-63.9)	36.7 (16.6-50.6)	61.1 (41-76.8)
Disophenol 20%	88.7 (75.3 – 95)	49.3 (28.5 - 64.1)	50.1 (34.2-61.1)	60.1 (39.1-75.5)	30 (-1.6 – 52.5)	91.7 (84.3-95.5)	77.6 (72.4-82.2)	73.2 (62-81.4)	96.3 (92.2-98.4)	94.8 (87.5-98.5)
Closantel 10%	82.8 (66.1 - 91.6)	83.4 (73.6 - 90.5)	-1.27 (-27.9- 17.7)	52.4 (22.9-70.7)	18.5 (-22.2- 43)	-13.8 (-41.9- 9.08)	58.2 (50.6-65.5)	57.5 (43.2-68.8)	-28.1 (-57.3 - -3.8)	97.5 (91.5-99.6)
Fenbendazole 10%	91.4 (82.3 - 96.5)	97.4 (92.8 - 99.5)	85.2 (76.9-91.1)	54.8 (29.3-71.1)	91.5 (81.8-96.9)	76.2 (65.4-83.8)	88.4 (84.6-91.3)	91.7 (85.1-95.6)	91 (85.2-95.1)	97.8 (92.2-99.7)

Table 3. Efficacy (%) of different anthelmintic drugs against each identified genus of gastrointestinal nematode fourteen days after treatment in naturally infected beef cattle at ten farms in the state of Rio Grande do Sul, Brazil.

		Anthelmintic treatments and reduction percentage for each genus after treatment									
Farm	**Genus**	Ivermectin	Doramectin	Eprinomectin	Moxidectin	Levamisole	Albendazole	Fenbendazole	Closantel	Nitroxynil	Disophenol
1	Coop	0	0	0	100	0	1.2	0	0	0	0
	Haem	0	100	0	0	100	0	100	100	100	100
	Oesop	100	100	0	0	100	100	0	100	100	0
	Ostert	100	100	100	100	100	100	100	0	0	100
	Trich	100	100	100	100	0	72.1	100	100	100	35.8
2	Coop	78.7	28.7	71.2	47.4	82.4	10.55	100	23	48.7	47.2
	Haem	0	0	0	0	-	-	-	-	0	-
	Oesop	100	49.5	0	76.7	100	100	100	100	100	100
	Ostert	-	-	-	-	-	100	-	-	-	-
	Trich	100	0	0	0	0	72.1	-	100	100	0
3	Coop	10	16.6	79.2	40	40	30.8	0	50	0	0
	Haem	0	0	0	0	0	0	0	0	0	-
	Oesop	0	0	0	0	100	100	100	100	100	100
	Ostert	0	0	0	-	0	0	0	0	0	0
	Trich	100	71.4	33	78.7	7.1	25.8	36.5	39.2	85.7	78.5
4	Coop	26.31	58.4	34.2	26.4	23.2	28.9	34.2	0	45.1	0
	Haem	0	0	0	0	47.9	87.5	0	75	0	87.5
	Oesop	-	0	-	0	-	0	0	-	0	100
	Ostert	0	100	100	0	100	0	100	0	100	100
	Trich	0	100	0	51.6	0	0	0	0	0	0
5	Coop	0	0	0	0	0	0	100	0	0	0

	Haem	100	100	100	37.5	100	75	100	100	80.7	50
	Oesop	-	-	-	-	-	0	-	-	-	-
	Ostert	0	0	0	100	100	100	0	0	0	100
	Trich	16.6	16.6	100	100	100	66.6	100	100	48.7	0
6	Coop	54.7	11.7	76.4	1.9	11.7	41.1	0	0	0	78.9
	Haem	0	0	0	0	60	0	0	100	100	0
	Oesop	-	0	0	0	-	-	-	-	-	-
	Ostert	-	-	-	78.7	-	50	-	-	-	-
	Trich	58.7	78.5	39.2	-	75	-	70.2	60.7	71.4	87.2
7	Coop	0	0	0	28.5	0	30	0	0	0	0
	Haem	0	31.8	100	0	100	0	72.7	77.2	59	49.5
	Oesop	0	0	100	0	100	0	100	100	100	100
	Ostert	100	100	0	100	100	100	100	0	0	100
	Trich	100	0	0	100	0	0	0	0	100	100
8	Coop	0	33.3	47.1	24.1	6.6	0	100	0	33.3	7.3
	Haem	24.1	0	0	0	0	100	100	100	100	0
	Oesop	0	0	0	0	100	100	100	100	100	0
	Ostert	-	-	-	-	-	-	-	-	-	-
	Trich	0	0	-	0	0	-	-	-	0	-
9	Coop	0	0	0	0	0	0	0	0	0	0
	Haem	100	25	0	0	100	100	100	75	100	0
	Oesop	100	100	71.4	25	100	100	100	100	100	100
	Ostert	-	-	-	-	-	-	-	-	-	-
	Trich	-	-	-	-	-	-	-	-	-	-
10	Coop	0	0	25.9	NL	3.8	0	6.2	0	0	0

Haem	30.7	61.5	0	NL	100	37.5	42.3	100	76.9	87.6
Oesop	0	20	0	NL	100	37.5	100	100	20	100
Ostert	-	-	-	NL	-	-	-	0	-	-
Trich	-	-	-	NL	-	0	0	0	-	-

Coop: *Cooperia* spp.; Haem: *Haemonchus* spp.; Oesop: *Oesophagostomum* spp.; Ostert: *Ostertagia* spp.; Trich: *Trichostrongylus* spp.; NL: no viable larvae after treatment (D+14)

Table 4. Efficacy (mean (%) and standard deviation) of anthelmintic drugs against each genus of gastrointestinal nematode fourteen days after treatment in naturally infected beef cattle at ten farms in the state of Rio Grande do Sul, Brazil

Compounds	Genera				
	Cooperia spp.	*Haemonchus* spp.	*Oesophagostomum* spp.	*Ostertagia* spp.	*Trichostrongylus* spp.
Ivermectin 1%	19.4 (±27.5)	23 (±41.6)	42.8 (±53.4)	40 (±54.7)	46.9 (±47.9)
Doramectin 1%	14.8 (±19.8)	31 (±41.2)	29.9 (±42.9)	60 (±54.7)	45.8 (±45.8)
Eprinomectin 0.5%	33.4 (±42.9)	20 (±42.1)	21.4 (±40.4)	40 (±54.7)	38.9 (±44.7)
Moxidectin 1%	29.8 (±31.5)	4.1 (±12.5)	12.7 (±27.3)	60 (±54.7)	63.6 (±42.5)
Levamisole 7.5%	16.7 (±26.4)	67.5 (±43.0)	100(0)	66.6 (±51.6)	22.7 (±40.5)
Albendazole 15%	14.2 (±16.5)	50 (±48.0)	59.7 (±49.1)	60 (±54.7)	26.8 (±31.7)
Fenbendazole 10%	34.0 (±46.6)	57.2 (±46.9)	75 (±46.2)	60 (±48.9)	43.8 (±46.2)
Closantel 10%	7.30 (±16.6)	80.8 (±32.5)	100 (0)	0 (0)	37.4 (±44.6)
Nitroxynil 34%	12.7 (±20.8)	61.6 (±44.5)	77.5 (±42.0)	40 (±54.7)	38.2 (±43.3)
Disophenol 20%	13.3 (±27.3)	46.8 (±42.6)	75 (±46.2)	80 (±44.7)	43 (±44.8)
Overall efficacy means (%)	**19.5**	**44.2**	**59.4**	**50.6**	**40.7**

Table 5. Percentage of EPG reduction (and 95% confidence interval) calculated by the fecal egg count reduction test (FECRT) fourteen days after treatment with anthelmintic combinations in beef cattle naturally infected by gastrointestinal nematodes at farms 1, 3, 7, and 8 in the state of Rio Grande do Sul, Brazil.

Anthelmintic combination	Reduction of EPG after treatment on each farm			
	1	**3**	**7**	**8**
Moxidectin 1% + Levamisole 7.5%	98.1 (93.5-99.8)	96.5 (93.1-98.6)	99.1 (94.6-100)	98.9 (95.2-100)
Moxidectin 1% + Albendazole 15%	87 (76.8-93.7)	93.1 (87.7-96.3)	78 (64-87.3)	95.7 (89.4-98.9)
Albendazole 15% + Closantel 10%	63 (47.4-74)	61.7 (50-72.2)	64.7 (44-78.2)	51.4 (28.6-67.8)
Doramectin 1% + Closantel 10%	67.1 (49.6-78.5)	54.9 (40.5-65.5)	71.9 (56.2-82.5)	66.3 (46.1-77.9)
Doramectin 1% + Fenbendazole 10%	87.9 (77.4-93.7)	93.1 (89.2-96.4)	89.2 (78.1-95.4)	98 (91.2-99.8)
Levamisole 7.5% + Closantel 10%	94.3 (88.7-97.9)	87.2 (79.9-92.4)	81.2 (67-90)	91.8 (83.3-96.4)

Table 6. Efficacy (%) of anthelmintic combinations against each genus of gastrointestinal nematode fourteen days after treatment in naturally infected beef cattle herds at farms 1, 3, 7, and 8 in the state of Rio Grande do Sul, Brazil.

		Anthelmintic treatment and percentage reduction of each genus					
Farm	**Genus**	Moxi + Leva	Moxi + Albe	Albe + Clos	Dora + Clos	Dora + Fenb	Leva + Clos
1	Coop	NL	0	0	0	42.8	0
	Haem	NL	100	100	100	0	100
	Oesop	NL	100	100	100	100	100
	Ostert	NL	-	-	-	-	-
	Trich	NL	-	-	-	-	-
3	Coop	100	23	0	0	0	0
	Haem	100	0	100	0	100	100
	Oesop	100	41.1	100	100	100	100
	Ostert	100	0	87.6	100	100	25.6
	Trich	0	47	100	29.3	100	0
7	Coop	100	16.2	35.1	0	0	100
	Haem	100	100	100	100	100	100
	Oesop	0	100	0	0	100	0
	Ostert	-	-	-	-	-	100
	Trich	-	0	-	-	-	-
8	Coop	NL	0	0	0	100	0
	Haem	NL	100	100	100	100	0
	Oesop	NL	100	-	-	100	100
	Ostert	NL	-	-	-	-	-
	Trich	NL	-	-	-	-	-

Moxi+Leva = moxidectin 1% (0.2 mg/kg, subcutaneous) + levamisole 7.5% (3.75 mg/kg, subcutaneous), Moxi+Albe = moxidectin 1% (0.2 mg/kg, subcutaneous) + albendazole 15% (3.4 mg/kg, subcutaneous), Albe+Clos = albendazole 15% (3.4 mg/kg, subcutaneous) + closantel 10% (10 mg/kg, oral), Dora+Clos = doramectin 1% (0.2 mg/kg, subcutaneous) + closantel 10% (10 mg/kg, oral), Dora+Fenb =doramectin 1% (0.2 mg/kg, subcutaneous) + fenbendazole 10% (5 mg/kg, oral), Leva+Clos = levamisole 7.5% (3.75 mg/kg, subcutaneous) + closantel 10% (10 mg/kg, oral). Coop: *Cooperia* spp.; Haem: *Haemonchus* spp.; Oesop: *Oesophagostomum* spp.; Ostert: *Ostertagia* spp.; Trich: *Trichostrongylus* spp.; NL: no viable larvae after treatment (D+14).

Table 7. Efficacy (mean (%) and standard deviation) of anthelmintic combination against each genus of gastrointestinal nematode fourteen days after treatment in naturally infected beef cattle at farms 1, 3, 7, and 8 in the state of Rio Grande do Sul, Brazil.

Anthelmintic combinations	Genera				
	Cooperia spp.	*Haemonchus* spp.	*Oesophagostomum* spp.	*Ostertagia* spp.	*Trichostrongylus* spp.
Moxidectin 1% + Levamisole 7.5%	100 (0)	100 (0)	75 (±70.7)	100 (0)	0 (0)
Moxidectin 1% + Albendazole 15%	9.8 (±11.6)	75 (±50)	85.2 (±29.4)	0 (0)	23.58 (±33.2)
Albendazole 15% + Closantel 10%	8.7 (±17.5)	100 (0)	75 (±57.7)	87.6 (0)	100 (0)
Doramectin 1% + Closantel 10%	0 (0)	75 (±50)	75 (±57.7)	100 (0)	29.4 (0)
Doramectin 1% + Fenbendazole 10%	35.7 (±47.3)	100 (±50)	100 (0)	100 (0)	100 (0)
Levamisole 7.5% + Closantel 10%	25 (±50)	6.6 (±50)	75 (±50)	25.7 (±52.6)	0 (0)
Overall efficacy means (%)	**29.8**	**76.1**	**80.8**	**68.8**	**44.7**

3 ARTIGO 2

(Artigo metido à revista "Small Ruminant Research")

Anthelminthic resistance of gastrointestinal nematodes in sheep to monepantel in the central region of the state of Rio Grande do Sul, Brazil

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Abstract. Sheep's sanity can be affected by various disorders. Among such disorders are the parasitic infections caused by gastrointestinal nematodes. Given the numerous reports of anthelminthic resistance of gastrointestinal nematodes, the objective of this study was to evaluate the efficacy of monepantel (Zolvix ®) against the gastrointestinal nematodes in naturally infected lambs at two farms in the state of Rio Grande do Sul. The lambs at farm 1 were separated into two groups, a non-treated control group (n = 17) and an experimental group that was treated with monepantel (n = 17). Similarly, at farm 2, there was a control group (n = 10) and a treatment group that received the same drug (n = 20). When the study began, a count of eggs per gram of feces (EPG) was conducted for each animal. The fecal samples were collected one day before (D-1) and nine days after (D+9) treatment began. The use of monepantel was 25.8% and 78.4% effective at farms 1 and 2, respectively. The genera

Haemonchus, *Trichostrongylus*, and *Cooperia* were resistant to the treatment. Monepantel did not effectively control the parasitic populations present on either of the farms studied in Rio Grande do Sul, Brazil.

Keywords: small ruminants, gastrointestinal helminths, EPG, efficacy

1. Introduction

Brazil has a population of over 17.6 million sheep, with the northeast and mid-west regions being the largest producers of sheep in the country. Particularly in the southern region, the state of Rio Grande do Sul plays a prominent role, holding more than 4 million heads of the most diverse breeds of sheep (IBGE, 2014).

Among the various health problems that affect these small ruminants, parasitic infections caused by gastrointestinal nematodes are one of the main obstacles faced by sheep farmers (Sczesny-Moraes et al., 2010). Given the high frequency of treatments that these animals are subjected to, several studies have reported the ineffectiveness of various chemical groups at combating these nematodes. Such reports include the work done by Cezar et al. (2010), Borges et al. (2015), and Bichuette et al. (2015) in the states of Rio Grande do Sul, Bahia, and São Paulo, respectively.

In the year 2012, the drug monepantel (Zolvix ®), from a group of amino-acetonitrile derivatives (AADs), was launched in the Brazilian market. Despite this being a new molecule, in recent years, there have been reports of parasitic resistance in several regions worldwide, including Brazil, (Scott et al., 2013; Mederos et al. 2014; Van den Brom et al. 2015, Cintra et al., 2015).

In light of these reports, the present study aimed to verify the efficacy of monepantel (Zolvix ®) in controlling gastrointestinal nematodes in naturally infected lambs on two farms in the central region of Rio Grande do Sul, Brazil.

2. Materials and methods

2.1 Farms and animals

This study was conducted at two farms located in the central region of Rio Grande do Sul, which is located in the municipality of São Martinho da Serra (29° 32' 03.0"S, 53°51'24.04"W). Farm 1 performs the breeding of cattle, horses, and sheep, and has an area of 1800 hectares**.** This farm features a flock of 1000 Texel and Suffolk sheep of various ages and sexes. The control of gastrointestinal parasites is based on the execution of annual efficacy tests of various anthelminthic treatments. The treatments are administered when the animals displays clinical signs suggestive of parasitosis. These signs include apathy, weight loss, a drop in food consumption, emergence of submandibular edema, paleness of mucous membranes, and an increase in the egg count per gram of feces (EPG). Selective treatments are not performed. According to the results obtained through previous efficacy tests, the gastrointestinal nematodes present in this flock have been found to be resistant to different anthelminthic compound, such as ivermectin, moxidectin, nitroxynil disophenol, levamisole, albendazole, and closantel, with the genera *Haemonchus*, *Cooperia,* and *Trichostrongylus* being the most resistant to treatment**.**

Similarly, farm 2 performs the breeding of equines, cattle, and sheep, maintaining 265 animals of this last species, that have an area of 150 hectares to graze. The flock of sheep is composed of Texel and Texel–Crioula cross breeds, and the control of gastrointestinal parasites is carried out in a non-selective way, based on the results of eventual efficacy tests. The treatments are administered at intervals of 30 to 45 days or as the appearance of the same clinical signs described earlier. According to the last efficacy test performed at this farm, some compounds of the avermectin group, moxidectin, levamizole, fenbendazole, and closantel were incapable of controlling the gastrointestinal parasite infection, with the

genera *Haemonchus*, *Trichostrongylus*, *Cooperia*, and *Ostertagia* being the most resistant to treatment.

To increase the efficacy of treatment on both farms, and given the availability of monepantel in the Brazilian market, it was introduced to control gastrointestinal parasites in young animals, as this age group is the most sensitive to this type of infection. However, after the second and fourth applications of the treatment at farms 1 and 2, respectively, the farms observed that some animals continued to present clinical signs suggestive of parasitosis by gastrointestinal nematodes, which indicated the inefficacy of this compound.

2.2 Experimental groups and animals treatment

Fecal samples from naturally infected lambs, which were approximately six months of age, were collected at both farms 30 days after the last anthelminthic treatment was administered. At farms 1 and 2, samples were collected from 126 (farm 1) and 75 (farm 2) female and castrated male sheep of all breeds present on those farms.

Fecal samples were collected one day before (D-1) and nine days (D+9) after treatment began, a practice adopted from previous studies conducted by Mederos et al. (2014) and Van den Brom et al. (2015). All samples were stored in individual plastic bags and refrigerated until processing, which was done immediately after all samples were collected.

The count of eggs per gram of feces (EPG) was performed using a modified McMaster technique, with a sensitivity of 100 EPG. Therefore, 2 g of homogenized feces were mixed with 58 ml of a saturated solution, filtered, and transferred to a McMaster chamber for microscopic identification and for calculating the EPG. The animals that presented EPG $\geq$ 200 on D-1 were selected and divided into two groups forming randomized blocks. In farm 1, one control group (n=17) was used to monitor the natural changes of EPG throughout the experimental period (Lyndal-Murphy et al. 2014; Neves et al. 2014) and other was an

experimental group (n=17) treated with monepantel (Zolvix ®, Elanco) at a dosage of 2.5 mg/kg, the product's recommended dosage. All animals in the treated group were weighed, and they received monepantel orally with a syringe at a dose corresponding to their body weight, whereas the control group did not receive any type of treatment.

Similarly, at farm 2, the animals were selected according to the same criteria as those used at farm 1, forming a control group (n=10) and a treated group (n=20). The treatment of the animals was performed using the same processes as well.

Cultures of feces were performed at days D-1 and D+9 using a pool of animal fecal samples, which were mixed with sterile sawdust and incubated for seven days at 27 ºC and 80% atmospheric humidity, according to the recommendations of Coles et al. (2006). After incubation, 100 third stage larvae, both from the control groups and the treated groups of both farms, were identified by genera following the criteria described by Van Wyk and Mayhew (2013).

The use of animals was approved by the Ethics in Animal Experimentation of the Federal University of Santa Maria, under protocol no. 8088190815.

2.3 Analysis and interpretation of results

As recommended by Neves et al. (2014), the pre and post-treatment EPG counts of the treated group were used to determine the efficacy of the monepantel treatment. For this purpose, we used the approach described by Torgerson et al. (2014), which is available at http://www.math.uzh.ch/as/index.php?id =254& L=1.

The efficacy of treatment, according to the genera of parasites identified in the culture of larvae from days D-1 and D+9, was determined using the formula: PR= 100 x (1 - PERfinal/PERinitial). In this equation, PR is the percentage of reduction by

genera, PERinitial and PERfinal are the percentages of each gender one day before (D-1) and 9 days (D+9) after treatment began, respectively (Coles et al., 1992; Coles et al., 2006).

The status of anthelminthic resistance was interpreted according to the recommendations of Lyndal-Murphy et al. (2014), based on the guide of the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles et al., 1992), which analyses the reduction percentage of the EPG and the upper limits (UL) and lower limits (LL) of the confidence interval (CI) to 95%. Thus, the treatment was classified as: effective (percentage of reduction of EPG and UL 95% equal or superior to 95% and 95% LL above 90%); ineffective (percentage reduction and UL95% below 95% and LL95% below 90%; or inconclusive (none of the previous criteria filled).

3. Results

The arithmetic means, maximum and minimum EPG, and EPG reduction percentage of groups treated with monepantel are presented in Table 1. The genera of parasites identified after the fecal cultures from D-1 were *Haemonchus* (46%), *Trichostrongylus* spp. (38%), and *Cooperia* (16%) at farm 1 and *Trichostrongylus* (54%), *Haemonchu*s spp. (32%), *Cooperia* (10%), and *Ostertagia* (4%) at farm 2. Nine days after the monepantel treatment (D+9), the reduction percentages and the lower and upper limits of 95% CI were evaluated. The results indicated that this treatment was ineffective at controlling the parasitic population present at the two farms. The genera *Trichostrongylus* (77%), *Haemonchus* (15%) and *Cooperia* (8%) were identified in the fecal cultures at farm 1 after treatment. Additionally, at farm 2, larvae of the parasites of genera *Haemonchus* (64%), *Trichostrongylus* (24%) and *Cooperia* (14%), were present in the fecal samples collected from the treated group on day D+9. Therefore, the effectiveness of the treatment on the genera present at farm 1 was 30.4% for *Haemonchus*; 100% for *Cooperia*;

0% for *Trichostrongylus*, and at farm 2 was 100% for *Ostertagia*, 55.5% for *Trichostrongylus*; 0% for *Haemonchus* and *Cooperia*. As for the animals in the control group, with the exception of one animal at farm 1 that had its EPG zeroed on D+9, the maintenance or increase of the parasitic infections were observed in the sheep from both farms. The clinical signs, such as apathy and anemia of mucous membranes, worsened in some animals when the second collection of fecal samples was performed. Additionally, the same genera of parasites were observed in the fecal cultures of D+9 as those observed in the samples collected on D-1.

4. Discussion

The resistance of gastrointestinal nematodes in small ruminants to treatment can be observed in several regions of the world, including in Brazil (Duarte et al., 2012; McMahon et al., 2013; Martínez-Valladares et al., 2015). Little et al. (2010) have argued that the treatment of the animals with effective anthelminthic therapies, used in a strategic way and in conjunction with other control practices, is the best option for producers. However, studies have shown that parasite resistance has gradually increased. The continuing lack of information concerning the treatment of parasite-infected animals has led to the inefficacy of the compounds belonging to the three major classes of broad-spectrum anthelminthic treatments (Sutherland et al. 2008). Consequently, farm and flock owners have suffered serious consequences, including, in some cases, the dissolution of their flocks and properties (Blake & Coles, 2007).

The drug monepantel belongs to a group of amino-acetonitrile derivatives (AADs), which represent a new class of anthelminthic therapy. This compound was launched in the year 2009, reaching the Brazilian market in 2012, and consequently providing an alternative treatment for farmers (Hosking et al., 2008). However, in a similar manner to data obtained by Mederos et al. (2014) and Scott et al. (2013) in Uruguay and New Zealand, respectively,

parasitic populations that were resistant to monepantel were observed on the farms featured in the studies four years after the product became available in the market.

After seventeen applications of this anthelminthic treatment over the course of two years, Scott et al. (2013) observed strains of *T. circumcincta* and *T. colubriformis* that were resistant to the treatment in goats and sheep. However, the results obtained in our study suggest that the appearance of resistant strains occurs after fewer generations than observed in the study performed by Scott et al. (2013). The presence of populations of *Trichostrongylus* spp., *Haemonchus* spp.
and *Cooperia* spp. resistant to treatment were observed after only two and four applications of this principle in its therapeutic dosage, at farms 1 and 2, respectively. These data mirror those obtained by Cintra et al. (2015), who found the selection of strains of *Trichostrongylus colubriformis* following the use of monepantel in a non-suppressive treatment and involving the selective treatment of animals over a period of just five months.

One of the reasons for the apparent resistance of these gastrointestinal nematodes populations to monepantel, may be due to the low presence of larvae in refuge, or in other words, the larvae that had no exposure to the anthelminthic treatments, because there was no execution of selective treatments at either farm, and the treatments were executed in a suppressive form (Kenyon et al. 2009). This practice leads to rapid selection within the nematode populations because, even if a small parcel survive an effective treatment, this will be the unique stock of larvae available for the reinfection of flock (Busin et al. 2013). However, Bartley et al. (2015) argue that aspects such as prior exposure to anthelminthic compounds, inherent sensitivity to compounds in functional dose-limiting species, the initial frequency of potential genes for resistance within a population, the nature of the genetic heritage (dominant/recessive), and the impact of non-specific mechanisms of resistance on the survivability of certain isolated individuals, hinder the estimates of what

may occur within certain parasitic populations after the anthelminthic treatment is administered to the animals.

Combinations of antiparasitic principles could restore the effectiveness of the treatments on the studied farms, as was demonstrated in a study conducted by Cezar et al. (2011). However, similar to conventional treatments, the long-term use of such combinations can become ineffective as the nematode populations are resistant to all classes of available drugs (Cezar et al. 2010). Regardless, alternative methods of control must be integrated to productive systems. These new methods should be more effective than combined allopathic treatments and help to slow the development of resistance (Waller, 2006; Torres-Acosta & Hoste, 2008).

The results of this study, opposite the historic anthelminthic resistance to other active principles at both farms, confirm the need for the establishment of control programs based on clinical and epidemiological criteria, and the need for frequent monitoring of the efficacy of such treatments (Cezar et al. 2010). In this context, according to the recommendations of Waller (2006) and Molento et al. (2013), alternative methods that will lead to a reduction in the frequency of treatments should be adopted. Such methods could include the selection of less susceptible animals and the alternation between agricultural and livestock farming activities. This may slow down the development of parasite resistance and encourage the lambs' development.

5. Conclusions

The resistance of gastrointestinal nematodes present in sheep ishas been gradually worsening after the latest compound launched in the Brazilian market, the monepantel has proved to be ineffective at both properties studied in the state of Rio Grande do Sul. Studies

aimed at understanding the molecular mechanism of the development of resistant parasitic populations to the monepantel treatment are necessary in order to develop strategies to delay the appearance of new cases of resistance to this compound and increase its service life.

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Table 1. Results of eggs per gram of feces (EPG): arithmetic average, maximum and minimum values of EPG and reduction of EPG with upper and lower values of the confidence interval (CI) to 95%, before (D-1) and after (D+9) monepantel treatment, of lambs naturally infected in the state of Rio Grande do Sul, Brazil.

	Day-1		Day + 9		
	Average	Epg min-max	Average	Epg min-max	Reduction (95% CI)
Prop. 1					
Control (n=17)	1006,3	200-5500	1629,4	0-10400	NA
Monepantel (n=17)	10835	2000-24500	7970,6	2600-16600	25.8 (20,1-30,7)
Prop.2					
Control (n=10)	536,3	200-1900	1590,1	400-5200	NA
Monepantel (n=20)	1170	200-5700	240	0-2300	78.4(68,5-83,7)

NA: not apply; CI: confidence interval

4 CONCLUSÕES

A resistência parasitária múltipla aos fármacos com ação anti-helmíntica, provavelmente, encontra-se disseminada em rebanhos de ruminantes no Rio Grande do Sul, havendo a necessidade eminente da adoção de medidas de controle, de forma a se recuperar a eficácia dos tratamentos, sob pena desta problemática atingir um patamar tão grave, a ponto de inviabilizar economicamente a manutenção de alguns desses rebanhos.

A combinação de princípios ativos com mecanismo de ação quimicamente distintos, disponíveis separadamente no mercado, tendem a resultar em maior eficácia dos tratamentos em populações de nematódeos gastrintestinais multirresistentes. Porém, ressalta-se a importância da execução de testes de eficácia e cultura de larvas, para a correta escolha dos fármacos que irão compor as combinações, conforme cada caso.

A resistência parasitária de nematódeos gastrointestinais de ovinos está gradativamente agravando-se, uma vez que a ineficácia do último princípio ativo lançado no mercado brasileiro foi comprovada e, provavelmente, deve estar presente em mais rebanhos no estado do Rio Grande do Sul, especialmente naqueles de criação intensiva.

Manejos alternativos, como o pastejo com outras espécies de herbívoros, rotação entre áreas de pecuária e lavoura, entre outras práticas, poderiam ser gradativamente incorporadas nas propriedades do Rio Grande do Sul, juntamente com a realização periódica de testes de eficácia, na tentativa de se manter o bom desempenho dos animais e a viabilidade dos anti-helmínticos dentro das fazendas por mais tempo.

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