



Field and Molecular Evaluation of Anthelmintic Resistance of Nematode Populations from Cattle and Sheep Naturally Infected Pastured on Mixed Grazing areas at Rio Grande do Sul, Brazil

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Abstract

Background Reports of a lack of efficacy of most of the anthelmintic compounds for ruminants associated with the long-time necessity for creating new molecules have stressed the urgency to adopt alternative methods to control gastrointestinal parasites infection, such as strategies of sharing grazing areas. Therefore, this study aimed to evaluate nematode populations affecting cattle and sheep that share grazing areas before and after treatment with different anthelmintic compounds, and investigate the efficacy of anthelmintic treatment in these naturally infected ruminants at farms in the state of Rio Grande do Sul, Brazil.

Methods The presence of co-infections by *Haemonchus* species was investigated by polymerase chain reaction (PCR) for groups treated with a benzimidazole. Farms were selected by: farmers' consent, presence of 42–60 (or more) calves and sheep per farm with counts of ≥ 200 eggs per gram of feces (EPG), availability of calves and lambs aging from 6 to 9 months, absence of anthelmintic treatment for both species for 60 days before the experimental period, and shared grazing areas between this species on each farm. Animals were distributed into six treatment groups for each ruminant species per farm and treated with: ivermectin, doramectin, moxidectin, levamisole, albendazole, and closantel.

Results Levamisole was the most effective anthelmintic compound for both ruminant species. In general, *Cooperia* spp., *Haemonchus* spp., and *Trichostrongylus* spp. were the genus present after tested treatments that were ineffective. PCR showed the presence of *Haemonchus* species co-infections between cattle and sheep.

Conclusion Therefore, this study demonstrated the similarity between nematode population, the presence of multi-resistant nematodes, and the presence of *Haemonchus* species co-infections affecting different ruminant species that share pastures.

Keywords Drug resistance · FERCT · PCR · *Haemonchus*

Introduction

Gastrointestinal parasites from the strongyles group are an important issue related to economic losses in tropical and sub-tropical areas of the world [2]. To avoid losses related to parasitological infection on ruminants, many farmers use frequent application of broad-spectrum anthelmintics mainly macrolactones, benzimidazoles, and imidazothiazoles

chemical groups, which has led to the emergence and spread of parasitic resistance [26, 35].

Reports of a lack of efficacy of most of the anthelmintic compounds for ruminants associated with the long time necessity for creating new molecules have stressed the urgency to adopt alternative methods to control gastrointestinal parasites infection, such as pasture management and decontamination of grazing areas strategies [8, 25]. Particularly, this last strategy is one of the most employed in the state of Rio Grande do Sul, in grazing areas different animal species as horses, cattle, and sheep are pastured in the same areas, which provide decontamination of the grazing areas based on the specificity of gastrointestinal nematodes [36]. Studies have demonstrated promising results from this kind of practice; in this context, Marley et al. [23] have found

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better results on the development of lambs that were kept on the same area than adult cattle. On the other hand, some studies as have demonstrated the possibility of crossed infections with some important nematodes as *Haemonchus contortus*, *Haemonchus placei*, and *Cooperia* spp. due to this kind of practice [27, 31].

Therefore, the aims of this study were to evaluate the nematode populations affecting cattle and sheep that share grazing areas before and after treatment with different anthelmintic compounds and investigate the efficacy of the anthelmintic treatment in these two species of ruminants in naturally infected animals at farms in the state of Rio Grande do Sul, Brazil. In addition, groups of cattle and sheep treated with a benzimidazole were investigated for the presence of co-infections by *Haemonchus* species.

Materials and methods

Animals and farms

The present study was performed on seven farms located in seven counties of the Rio Grande do Sul state in southern Brazil: São Gabriel, São Martinho da Serra, Dilermando de Aguiar, Bagé, Capão do Cipó, São Francisco de Assis, and Santa Maria. Farms and herds were selected based on previous consent by the farms and by the extensive system used to raise both cattle and sheep. Also, the following technical criteria were considered: the presence of 42–60 (or more) calves and sheep per farm with counts of ≥ 200 eggs per gram of feces (EPG); availability of *Bos taurus/Bos indicus* crossbred calves and lambs of any breed aging from 6 to 9 months; the absence of anthelmintic treatment for both species for 60 days before the experimental period; and areas grazed simultaneously by these species on each farm. Initially, all animals that met these criteria were included in the study; therefore, animals with fewer than 200 EPG before treatment were excluded. Animals were kept in the same grazing area before and during the study on each farm. The use of animals was approved by Committee of Ethics in Animal Experimentation of the Federal University of Santa Maria under protocol no. 7650140817.

Anthelmintic treatment

Six commercially available anthelmintic compounds were used to perform the efficacy tests on each farm. These compounds were administered by a veterinarian participant of the study following the manufacturer's recommendations: ivermectin 1% (200 mcg/kg, subcutaneous, Ivomec, Merial), doramectin 1% (200 mcg/kg, subcutaneous, Dectomax, Zoetis), moxidectin 1% (200 mcg/kg, subcutaneous, Cydec-tin, Zoetis), levamisole 7.5% (3.75 mg/kg, subcutaneous,

Ripercol L, Zoetis), albendazole 15% (3.4 mg/kg, subcutaneous, Agebendazol, Agener União), and closantel 10% (10 mg/kg, oral, Diantel, HIPRA). The oral treatments were performed without fasting.

Experimental groups and fecal analysis

All samples were collected directly from the rectum of each animal from both species 2 days prior to treatment (D – 2) and 14 days after (D + 14), according to recommendations of Coles et al. [13]. EPG counting was performed using a modified McMaster technique, with a sensitivity of 50 EPG. For both cattle and sheep, animals that had an EPG count ≥ 200 on D – 2 were selected. These animals were distributed into six randomized blocks (six blocks for cattle and six blocks for sheep) based on EPG at each farm, to balance the mean and the frequency distributions of EPG counting among groups before treatments. The number of calves and lambs in each experimental group ranged from 7 to 10 animals depending on available infected animals at each farm; therefore, the number of animals used per farm was: 56 calves and 42 sheep (farm 1), 48 calves and 48 sheep (farm 2), 60 calves and 60 sheep (farm 3), 48 calves and 60 sheep (farm 4), 60 calves and 60 sheep (farm 5), 52 calves and 48 sheep (farm 6), and 60 calves and 48 sheep (farm 7).

Larval cultures were made on each collection day from fecal samples of calves and lambs in each experimental group. Pooled feces were mixed with sterile wood shavings and stored for larvae cultures (moisturized daily with sterile water under incubation for 7 days at 22–27 °C and 80% humidity), according to the recommendations of Coles et al. [13]. After incubation, larvae were recovered by baermanization, and 100 third-stage larvae in each culture were identified (by genera) following the criteria described by Van Wyk and Mayhew [39].

Statistical analysis and interpretation of the results

To calculate the efficacy based on the reduction in EPG, in both species, pre- and post-treatment EPG counts were used. For this, the approach described by Torgerson et al. [38] was used (available at <http://shiny.math.uzh.ch/user/furrer/shiny-as/shiny-eggCounts/>). This 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.

For each genera of gastrointestinal nematode identified, the efficacy of the treatments was also estimated. The proportion of each genus of nematode in the larvae cultures at D – 2 and D + 14 from both animal species was considered using the following formula: $PR = 100 \times (1 - PER_{final}/PER_{initial})$, where PR is the percentage of reduction by genus, and

PER_{initial} and PER_{final} are the percentages of each genus before (D – 2) and 14 days after (D + 14) treatment, respectively [12, 13, 26]. Anthelmintic resistance status was evaluated according to the recommendation of Lyndal-Murphy et al. [22], based on the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines on anthelmintic resistance [12], 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 the 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 the 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).

Obtainment of larvae of third stage (L3) and adult *Haemonchus* specimens for PCRs

L3 larvae of *Haemonchus* spp. resistant to treatment to a benzimidazole (albendazole 15%, Agebendazol, Agener União) were obtained from the fecal cultures on D – 2 and D + 14 of the FECRT carried out on the first part of the study. Benzimidazole was selected based on its wide use in the farms for treatment of both ruminants' species [13, 29, 39]. After the identification of the cultures, larvae were transferred to Eppendorf tube with 1 ml of distilled water, and kept on freezer (– 10 °C) until the DNA extraction. All cultures before and after treatment from the ruminants present mixed infections by different genus of nematodes including *Haemonchus* spp. Although there was other genus present, *Haemonchus* spp. L3 was not individually separated from the others larvae present in the larvae pool once the primers used for the PCR analyses were specific for *Haemonchus* species.

As positive and negative PCR controls, we used individual adult specimens of *Haemonchus*; for this propose, we obtained samples of adult specimens of *Haemonchus contortus* from sheep raised in São Gabriel, and *Haemonchus placei* from cattle raised Uruguaiana, both counties of Rio Grande do Sul state. Adult specimens were kindly donated. The identity of both adult *Haemonchus* specimens was confirmed by PCR using the primers developed by Amarante et al. [3].

DNA extraction

DNA extraction of L3 larvae was done following the recommendations of Minho et al. [24], Testi [37], and Bekelaar et al. [5]. Briefly, suspensions of larvae cultures were incubated with 180 µl of 3.5% sodium hypochlorite during 5 min in petri dish for sheath removal. After that, all content of the petri dish was

transferred to 15 ml falcon tube and centrifuged at 3000 RPM for 5 min. This process was repeated four times with distilled water, to completely remove hypochlorite residues, after that, 300 µl containing the unsheathed larvae pellet was transferred to a cryotube and kept for 2 h on the liquid nitrogen vapor. Subsequently, cryotube was transferred into the liquid nitrogen during 5 min and transferred to 45 °C until completely thawed. This process was repeated 4–5 times and after that DNA concentration was measured on spectrophotometer Picodrop microliter. DNA from adult specimens was extracted from single larvae of *Haemonchus* with the PureLink™ Genomic DNA Mini Kit (Invitrogen, USA) according to manufacturer's instructions. After that, DNA concentration was also measured as described above.

PCR

Extracted DNA from L3 samples prior and after treatment with albendazole for both ruminants species and DNA from adults samples of *Haemonchus contortus* and *Haemonchus placei* were submitted to PCR using primers and reaction condition developed by Amarante et al. [3]. Briefly, two species-specific primers were used, one for *H. contortus* (*HcBotuF1* 5'-TGTCGAACACGAAACTCGTC-3' and *HcBotuR2* 5'-TGTTGTCTCTACCGCCCGAGT-3', and other for *H. placei* (*HpBotuF* 5'-CCAGACCCGAGACTCGCC-3' and *HpBotuR* 5'-CTGAAGGTAATGTCAAATTTCT-3'). PCR for each primer pair was performed separately; therefore, each sample was submitted to two PCRs, one for each primer pair separately. For *H. contortus* a 260 bp was expected, and for *H. placei* a 459 bp. Amplification was carried out in a 25 µl reaction mix containing 2.5 mM MgCl₂ (Promega), 1 × buffer (Promega), 10 µM each deoxynucleoside triphosphate (dNTP—KAPA BIOSYSTEMS), 20–50 ng genomic DNA, 1 U GoTaq® Hot Start Polymerase (Promega), 10 µM each primer. Cycling conditions for the primer pair HpBotuF/R were as follows: 95 °C for 5 min; followed by 35 cycles of 95 °C for 30 s, 58.5 °C for 15 s and 72 °C for 30 s; followed by 5 min at 72 °C and 4 °C to finalize. Primer pair HcBotuF1/R2 cycling conditions were largely the same, except the annealing time and temperature: 59 °C for 40 s. PCR products were electrophoresed on 1.5% agarose gels, colored with red gl 1/500 (UNISCENCE), and photographed under UV light (HoeferMacroVue UV-20) using a Canon PowerShot A640 (Canon, Melville, New York, USA).

Results and discussion

Table 1 presents the arithmetic means, standard deviation, minimum and maximum EPG counts, and percentages of each genus of gastrointestinal nematodes found on cattle

and sheep, respectively, prior to the treatment of each animal category at all farms. The presence of gastrointestinal nematodes with multiple resistance to the anthelmintic compounds used in this study was detected at all farms. Of all anthelmintic applied, levamisole 7.5% demonstrated the greatest percentage of reduction of EPG for both cattle and sheep, as demonstrated in Table 2. Most of the nematode populations evaluated from both ruminants presented multi-resistance to the treatments tested, which is a common result related to other studies as demonstrated by Sangison [7, 9, 10, 21, 33].

Larvae cultures from both ruminant species showed the presence of mixed infections before treatment, containing the genera *Cooperia*, *Haemonchus*, *Oesophagostomum*, *Trichostrongylus*, *Ostertagia*, and *Strongyloides*, with the last one being present only on sheep (Table 1). Most of the farms evaluated, *Cooperia* spp., *Haemonchus* spp., and *Trichostrongylus* spp. were resistant to different treatments applied on cattle, whereas *Oesophagostomum* spp. was the most susceptible. In sheep, *Haemonchus* spp., and *Cooperia* spp. presented greater resistance levels to anthelmintic compounds tested, while the genera *Ostertagia*, *Trichostrongylus*, and *Oesophagostomum* were the most susceptible (Tables 3 and 4). *Haemonchus* spp., and *Cooperia* spp. have already been associated with cases of resistance in several regions of Brazil and other countries,

as demonstrated in the studies made by Cezar et al. [9], Neves et al. [26], and Cristel et al. [14].

Due to the great number of resistance reports in gastrointestinal nematodes of ruminants, it is clear that there is a necessity for alternative methods of control. Although the mixed grazing with different ruminant species could be a good strategy for pasture decontamination, it is important to be aware of the possibility for cross-infection because some species can parasite both ruminants, such as *Trichostrongylus axei* and *Cooperia punctata* [15, 28, 34].

Age of the animals is another important factor in continuously mixed grazing areas, animals used in our study were aged 6–9 months, and it can be noted that mainly in sheep, age reflected in greater EPG counts before the treatment (Table 1). Similarly, Pinheiro [27] noted that the presence of calves and lambs in mixed grazing systems resulted in unsatisfactory results. Pinheiro et al. [28] found that good pasture decontamination was only obtained when lambs shared pasture with adult cattle and similar results were found by Fernandes et al. [15], Rocha et al. [30] and Santos [32].

Amplification pattern of PCR results is presented in Fig. 1 and in all PCRs the amplification patterns were similar to the ones obtained by Amarante et al. [3]. PCR results for both sheep and cattle from each farm are presented in Table 5 which demonstrated that some evaluated farms presented

Table 1 Arithmetic mean (AM, and standard deviation), minimum (MIN) and maximum (MAX) fecal egg counts and percentage of the different genera identified before the treatments (D – 2) in the feces

Farm	OPG			Genera of gastrointestinal nematodes of beef cattle (%)					
	MA(DP)	MIN	MAX	<i>Cooperia</i> spp.	<i>Oesophagostomum</i> spp.	<i>Haemonchus</i> spp.	<i>Ostertagia</i> spp.		
1	559.82 (487.7)	200	3200	72	7	21			
2	397.5(188.3)	200	900	16	2	80		2	
3	401.04 (223.2)	200	1150	14	4	82			
4	477.5(300.4)	200	1350	28	12	60			
5	515 (282.5)	200	1300	68	2	28		2	
6	378.4(361.4)	200	1900	33	6	61			
7	313.1(141.4)	200	750	, 40	22	38			

Farm	OPG			Genera of gastrointestinal nematodes of sheep (%)					
	MA(DP)	MIN	MAX	<i>Cooperia</i> spp.	<i>Oesophagostomum</i> spp.	<i>Haemonchus</i> spp.	<i>Ostertagia</i> spp.	<i>Trichostrongylus</i> spp.	<i>Strongyloides</i> spp.
1	604.76 (718)	200	3250	2		98			
2	425.83 (200.94)	200	900	12		78		10	
3	522.92 (431.2)	200	2100	8	14	48	10	20	
4	2213.4(2149.7)	200	9100		10	88		2	
5	1092.5 (688.8)	200	2750	2		92	2		
6	2238(2124.1)	200	10,050	2		96		2	
7	1781.81 (1475.5)	400	7359	8		64		6	22

of naturally infected beef cattle and sheep that share grazing areas continuously from seven different farms in the state of Rio Grande do Sul

Table 2 Percentage of EPG reduction (and 95% confidence interval) calculated by the fecal egg count reduction test (FECRT) 14 days after anthelmintic treatment in beef cattle and sheep naturally infected

by gastrointestinal nematodes that share grazing areas continuously at seven farms in the state of Rio Grande do Sul, Brazil

Farm	Anthelmintic compound					
	Ivermectin 1%	Doramectin 1%	Moxidectin 1%	Levamisole 7.5%	Albendazole 15%	Closantel 10%
1						
Cattle	27.9 (6.3–45.8)	5.8 (0.2–21.6)	86.1 (77.8–91.8)	93.1 (86.4–96.9)	27.2 (5.2–45.2)	4.5 (0.1–17.7)
Sheep	1.8 (0.06–8.5)	1.04 (0.03–5.4)	1.2 (0.04–5.92)	5.2 (0.2–19.9)	11.4 (0.68–31.1)	1.18 (0.05–2.52)
2						
Cattle	39.3 (11.9–58.1)	80.5 (73.4–86.2)	76 (65.7–83.8)	90.2 (80–95.8)	64.6 (45.4–78.3)	75.9(65.7–83.8)
Sheep	3.04 (0.11–13.7)	1.59 (0.05–7.75)	1.17 (0.04–5.86)	38.7 (14.7–56.1)	1.92 (0.07–9)	36.3 (10.8–55.1)
3						
Cattle	45.2 (18.7–64.1)	45.1 (17.9–64.1)	55.1 (30.4–71.3)	72.8 (54–85)	79.9 (64.9–89.6)	82.3 (68.5–91.1)
Sheep	67.9 (50.9–79.8)	86.1 (75.6–92.9)	99.2 (95.6–100)	39.9 (15.6–57.5)	82.9 (70.5–90.8)	57.3 (36.7–71.7)
4						
Cattle	1.89 (0.06–9.42)	5 (0.22–1.91)	34 (8.8–53)	91.7 (82.3–96.9)	74 (58.9–84.5)	87.3(76.6–93.7)
Sheep	9.49 (0.81–21.9)	49.9 (39.9–58.5)	3.53 (0.13–13)	98.1 (96.1–99.3)	20 (6.3–31.7)	73.3(67.5–78.2)
5						
Cattle	7.63 (0.36–28.8)	13.7 (0.67–38.3)	39.8 (8.62–63)	75.6 (54.4–88)	24.3 (2.28–50.2)	55.8 (25–75)
Sheep	2.1(0.09–9.3)	2.17(0.07–9.83)	2.25(0.1–10.2)	61.7(50.6–70.6)	1.62(0.06–7.3)	46.6 (33.1–57.5)
6						
Cattle	4.7(0.1–18.5)	4.6 (0.1–18.1)	49.3 (30.4–63.9)	91.7(84.7–96.1)	19.5(2.0–39.3)	30.5(8.4–48.7)
Sheep	59(51.2–65.9)	23.8(5.7–40.2)	11.3(1.3–22.6)	78.8(72.2–84.1)	7.05(0.3–18.2)	81.9(76.9–86)
7						
Cattle	13 (0.9–33.1)	4.7(0.1–20.8)	18.6(1.1–45.1)	90.8 (82.1–95.8)	59.2 (40.5–72.7)	50.1 (16.1–71.7)
Sheep	45.6 (34.3–55)	67.1 (59.9–73.2)	76.8 (70–82.2)	93 (89–95.9)	61.5 (53.6–68.2)	80.1 (74.5–84.7)

co-infections between the *Haemonchus* species in both ruminants before and after treatment.

Farms 1, 3, 5, and 6 presented similar results. At these farms, cattle were infected by both *Haemonchus* species before treatment, and the lack of efficacy of the treatment related with *Haemonchus* spp. was due to *H. contortus* resistant to albendazole. Also, at these farms, the flocks presented infections only by *H. contortus* pre- and post-treatment.

At farm 2, there were similar results as the ones described above for cattle but sheep presented infection by *H. contortus* and *H. placei* before the treatment. Different results were observed at farm 4 where all cultures from both ruminants presented only *H. contortus*, and at farm 7 sheep were infected only by *Haemonchus contortus* while for cattle PCRs were positive for both species of these helminths pre- and post-treatment.

The accurate identification of nematodes species by molecular technics is crucial not only for diagnosis but also for treatment and control of infections. Although *H. contortus* and *H. placei* are separated species based on morphology, genetic crossing studies and molecular markers, they are phylogenetically close and have very similar morphology, making accurate species differentiation challenging and time consuming [4, 11]. *H. contortus* and *H. placei* are

usually considered to predominantly infect small ruminants and cattle, respectively. *H. contortus* is known to be able to infect a large number of different ruminant hosts, as was observed at farm 4 [20]. Although *H. placei* appears to be somewhat less promiscuous, it can infect small ruminants as well as cattle, which was demonstrated at farm 2 [4, 16].

Results showed a worrying situation once they suggest an adaptation of these nematodes to hosts that usually are not of their preference at some farms, reflecting the lack of efficacy of anthelmintic treatments. Also, results obtained at farm 7 suggest the infection of cattle by both species or suggest the presence of hybrids infecting some animals [11]. According to Chaudhry et al. [11], the presence of co-infections, as was observed at some farms of the study, raises the possibility of interspecies hybridization occurring in the field.

Different studies by Pinheiro [27], Amarante et al. [4], Jacquet et al. [19], Achi et al. [1], Gasbarre et al., [16, 17], and Brasil et al. [6] demonstrated the occurrence of crossed infections of *H. placei* and *H. contortus* in sheep, goats, cattle and buffalo. Mixed grazing strategy with different species of ruminants is an alternative option for decontamination of pastures assisting the anthelmintic control; however, the strategy of shared grazing areas possibly may favor crossed infections as observed in our study [8, 25].

Table 3 Efficacy (%) of different anthelmintic drugs on each genus of gastrointestinal nematode 14 days after treatments on naturally infected beef cattle that share grazing areas with sheep, at seven farms in the state of Rio Grande do Sul, Brazil

Farm	Genus	Anthelmintic treatments and reduction percentage for each genus after treatment					
		Ivermectin 1%	Doramectin 1%	Moxidectin 1%	Levamisole 7.5%	Albendazole 15%	Closantel 10%
1	Coop	0	50	7.8	34	0	0
	Haem	11	0	0	0	44	52
	Oesop	0	0	0	100	0	0
	Trich				0		
	Ostert						
2	Coop	87.5	75	37.5	87.5	0	0
	Haem	0	0	20	33	15	70
	Oesop	0	0	0	0	0	0
	Trich		0				0
	Ostert	100	100	100	100	100	100
3	Coop	0	14.3	0	0	0	0
	Haem	14.6	12.1	31.7	18.2	58.5	59.7
	Oesop	50	0	0	100	100	100
	Trich						
	Ostert						
4	Coop	0	0	85.7	28.5	0	0
	Haem	40	26.7	46.7	55	90	93.3
	Oesop	83.3	0	0	0		100
	Trich			0	0		0
	Ostert						
5	Coop	9	9	9	0	0	0
	Haem	0	0	0	39.2	92.8	92.8
	Oesop	0	100	0	100	0	0
	Trich						
	Ostert	100	100	100	100	100	100
6	Coop	0	0	0	0	0	0
	Haem	87	36.6	45.1	71	70	100
	Oesop	60	0	80	100	0	60
	Trich				0		
	Ostert						
7	Coop	0	5	0	0	0	0
	Haem	0	0	0	26.3	0	100
	Oesop	54.5	90	45	81.8	90	100
	Trich						
	Ostert						

Coop *Cooperia* spp., Haem *Haemonchus* spp., Oesop *Oesophagostomum* spp., Ostert *Ostertagia* spp., Trich *Trichostrongylus* spp.

In the same way, results obtained by Pinheiro [27] and Pinheiro et al. [28] demonstrated the possibility of crossed infections involving *H. placei* and *H. contortus*, and unsatisfactory results when lambs and calves shared mixed grazing areas showing that age of cattle and sheep when keeping these animals together is fundamental for an effective pasture decontamination, which did not occurred on the farms that were evaluated in this study. Indeed, age was a criterion used by Rocha Sargison et al. [33] that obtained positive

results when evaluating alternately grazing by 2-year-old cattle and sheep.

Many studies have demonstrated nematodes populations resistant to different benzimidazoles treatments as well as other anthelmintic classes in cattle and sheep. However, most *Haemonchus* infections diagnosed on the field are not evaluated to the species level, which brings a lack of data on true species prevalence and the extent of co-infections [11, 26, 36].

Table 4 Efficacy (%) of different anthelmintic drugs on each genus of gastrointestinal nematode 14 days after treatments on naturally infected sheep that share grazing areas with beef cattle, at seven farms in the state of Rio Grande do Sul, Brazil

Farm	Genus	Anthelmintic treatments and reduction percentage for each genus after treatment					
		Ivermectin 1%	Doramectin 1%	Moxidectin 1%	Levamisole 7.5%	Albendazole 15%	Closantel 10%
1	Coop	0	0	0	100	100	0
	Haem	0	2	4	0	36.7	24
	Oesop						
	Trich				0	0	
	Ostert					0	0
	Stron					0	
2	Coop	33.3	100	50	66.6	100	
	Haem	0	0	0	0	0	0
	Oesop						56
	Trich	80	20	0	100	80	
	Ostert		0	0			0
	Stron						
3	Coop	0	50	100	50	0	73.3
	Haem	0	0	100	0	0	54.1
	Oesop	42.8	100	100	0	100	0
	Trich	90	90	100	70	100	90
	Ostert		90	100	0	0	
	Stron						
4	Coop					0	0
	Haem	0	0	0	16	4	91
	Oesop	100	100	100	100	100	20
	Trich	0	100	100	100	100	100
	Ostert		0		0	0	
	Stron						
5	Coop	66	100	100	66	66	100
	Haem	0	0	0	6.5	0	0
	Oesop				0		
	Trich		0		0		
	Ostert	100	100	100	100	100	100
	Stron						
6	Coop	0	0	0	0	0	0
	Haem	4.1	2	29.1	6.2	8	85.4
	Oesop						
	Trich	100	100	100	100	100	0
	Ostert						0
	Stron			0	0	0	0
7	Coop	75	100	75	100	100	75
	Haem	0	0	0	0	0	0
	Oesop				0		
	Trich	100	100	33	66.7	66.7	0
	Ostert				0	0	0
	Stron	100	100	100	100	100	100

Coop Cooperia spp., *Haem* Haemonchus spp., *Oesop* Oesophagostomum spp., *Ostert* Ostertagia spp., *Trich* Trichostrongylus spp., *Stron* Strongyloides spp.

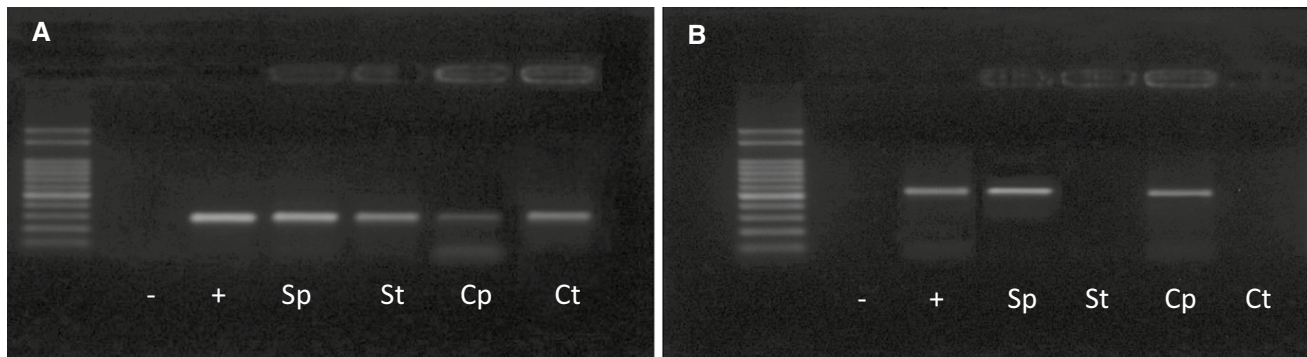


Fig. 1 Amplification pattern of PCRs for *Haemonchus* species identification using DNA extracted from fecal cultures of third-stage (L3) larvae prior and post-treatment with albendazole of sheep and cattle naturally infected by gastrointestinal nematodes that share grazing areas in Rio Grande do Sul state. **a** Amplification using HcBotu F/R primers. **b** Amplification using HpBotu F/R primers. First columns

demonstrate negative and positive controls, followed by DNA samples of L3 from sheep prior the treatment (Sp), L3 from sheep after the treatment with albendazole (St), L3 from cattle prior the treatment (Cp), and L3 from cattle after the treatment with albendazole (Ct). All samples were individually submitted to PCR for two primers for identification of *Haemonchus* species

Table 5 Molecular identification of *Haemonchus* species from third-stage larvae of cattle and sheep that share grazing areas before and after the treatment with a benzimidazole at seven farms in Rio Grande do Sul state, Brazil

Farm	Ruminant species	Before treatment		After treatment	
		<i>H. contortus</i>	<i>H. placei</i>	<i>H. contortus</i>	<i>H. placei</i>
1	Cattle	+	+	+	-
	Sheep	+	-	+	-
2	Cattle	+	+	+	-
	Sheep	+	+	+	-
3	Cattle	+	+	+	-
	Sheep	+	-	+	-
4	Cattle	+	-	+	-
	Sheep	+	-	+	-
5	Cattle	+	+	+	-
	Sheep	+	-	+	-
6	Cattle	+	+	+	-
	Sheep	+	-	+	-
7	Cattle	+	+	+	+
	Sheep	+	-	+	-

In many states of Brazil, not only at Rio Grande do Sul, farmers use same grazing areas for cattle and sheep without considering age of the animals. Therefore, a specific diagnosis is very important once these ruminants normally harbor mixed infections as observed in animals' fecal cultures of this study. Besides that, mixed infection possibly may favor the production of hybrids [18].

Also, the frequent anthelmintic treatment made without any technical criteria for selection of the appropriated drug, which is a common practice, could lead to genetic flux between populations, with further implications for the efficacy

of anthelmintic treatments, which could explain the results of the FERCT observed. This situation assistances interspecies transmission of resistance genes, as well as the introgression of genes involved in pathogenicity and host specificity [11].

Therefore, the results obtained in the present study showed that there were some similarities between the nematode population of sheep and cattle that share grazing areas continuously at the farms evaluated, with most of it showing multiple resistance to the anthelmintic drugs tested. In addition, at some farms, PCR tests showed the presence of co-infections of *Haemonchus* spp. or possible presence of hybrids infecting the animals. Co-infections demonstrated the adaptation of *Haemonchus* to different host at some farms with important implications in the success of anthelmintic treatments. Further studies aiming to evaluate the occurrence of co-infection and genetic flux of resistance between nematode species are necessary to identify the specific cause of drugs effectiveness and delay the appearance of new cases of drug resistance.

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Compliance with Ethical Standards

Conflict of Interest The authors of this manuscript have no financial or personal relationships with other people or organizations that could inappropriately influence or bias the content of the paper.

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