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Multidrug and multispecies resistance in sheep flocks from São Paulo state, Brazil

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ABSTRACT

The economic importance of sheep production is increasing worldwide simultaneously with the emergence of parasitic resistance. This study aimed to survey the current situation of management practices and parasite resistance in sheep flocks in São Paulo state, Brazil. A questionnaire was given to 35 sheep farmers to obtain information related to flock management practices. Of these flocks, 30 were submitted to the fecal egg count reduction test (FECRT) with at least one of the five following anthelmintics: albendazole, closantel, ivermectin, levamisole, and moxidectin, for comparison against an untreated control group. In the survey, the median number animals per flock was 301, mainly of the Santa Ines breed (in 75.8% of the flocks) and crossbred animals (in 54.5% of the flocks). The predominant farming system was semi-intensive (82.9%), using rotational grazing (80%). Selective treatment was based on FAMACHA grade (47.1%) and in clinical signs (41.2%). The most often applied anthelmintics were macrocyclic lactones (42.9–54.2% in the last three applications). Considering the anthelmintics employed in this study, 10.7% of the farms' flocks were resistant to three, 35.7% to four, and 53.6% to all five anthelmintics. The main helminth genera

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observed before and after treatments were *Haemonchus* sp. (75.8%) and *Trichostrongylus* sp. (19.1%), but all observed genera (*Cooperia* sp., *Oesophagostomum* sp., and *Strongyloides* sp.) were detected by the FECRT. Considering efficacy values less than or equal to 90% in the FECRT as resistant, 100% of flocks were resistant to albendazole and ivermectin, 96.6% to moxidectin, 92.9% to closantel, and 53.6% to levamisole. It is thus possible to conclude that multidrug resistance is widespread in sheep flocks in São Paulo state, Brazil, and this involves all prevalent helminth genera.

1. Introduction

The economic importance of sheep production is increasing worldwide. In Brazil, while the sheep population has remained constant over the past 40 years, in São Paulo, a state not historically associated with sheep production, the growth in the number of animals was nearly 360% between 1974 and 2010 (Sidra, 2010). In 2010, São Paulo was the tenth Brazilian state in number of sheep with over 467,000 animals (Sidra, 2010).

The main limiting factor for sheep farming is the high prevalence of gastrointestinal nematodes (GIN), of which *Haemonchus contortus* is the most prevalent and pathogenic. Parasitism by helminths causes losses related to reduced growth, weight gain and meat and wool quality, and occasionally causes death, not to mention the cost of treatment (Costa et al., 2007).

Besides this high prevalence, GIN control is hampered by parasitic resistance to anthelmintics, mainly because this is the main strategy employed for helminth control. Anthelmintic resistance of GINs in small ruminants is observed worldwide in various countries (Eddi et al., 1996; Maciel et al., 1996; Nari et al., 1996; Chandrawathani et al., 1999; Bartley et al., 2004; Waghorn et al., 2006; Díez-Baños et al., 2008) and Brazilian states (Farias et al., 1997; Cruz et al., 2010).

Four classes of broad-spectrum anthelmintics are commercially available in Brazil: benzimidazol, imidazotiazol, salicylanilide and macrocyclic lactones. Since the development of a new anthelmintic drug is a very slow process compared to the speed of resistance emergence (James et al., 2009), it is essential to detect the emergence of resistance early in order to extend the effectiveness of the available anthelmintics. Among the tests to detect anthelmintic resistance, the fecal count reduction test (FECRT) is the most widely employed. The FECRT is a practical and inexpensive test appropriate for all anthelmintic drugs and only requires the technician's ability to perform egg per gram (EPG) counts (Coles, 2005).

Unfortunately, easy access to anthelmintics and a lack of appropriate guidelines for their use contribute to the indiscriminate large-scale application of these drugs and have reduced the treatment effectiveness because of the increase in parasite resistance. Besides genetic conditions, the spread of resistance can be influenced by flock management practices (Barger, 1997). Thus, for early prevention and effective control of parasites it is necessary to obtain information about epidemiology and sheep management practices, to enable development of strategies to delay the establishment of resistance.

The objective of this study was to survey management practices employed in sheep flocks in São Paulo state, Brazil, and to assess helminth frequency and resistance to five anthelmintics (albendazole, closantel, ivermectin, levamisole, and moxidectin) by the FECRT.

2. Materials and methods

2.1. Management survey

Questionnaires were given at 35 sheep farms in São Paulo state, Brazil and the FECRT was applied to the flocks at 30 of these farms (Fig. 1) from November 2008 to July 2010. The survey obtained information on management practices, namely: number of animals and category, breeds, origin of the animals, replacement rate, attitude towards incoming animals, feed management (grazing area, rotational grazing and supplementation), and health management (anthelmintic brand and frequency, EPG count, FAMACHA method, and pasture rotation with other animal species).

2.2. Fecal egg count reduction test (FECRT)

Animals were selected for the FECRT (adapted from Coles et al., 2006) by the egg per gram (EPG) counts. Flocks with at least 80% of animals with EPG greater than to 200 were chosen. Using these criteria, it was possible to select 30 of the 35 flocks (flocks 1–30), of which 28 were evaluated for the 5 anthelmintics, 1 only for albendazole and moxidectin (flock 28), and 1 only for albendazole (flock 4), in addition to a control group.

In each flock, animals with the highest EPG, same breed, category and gender were selected and randomly distributed into groups of 10. Despite the differences in animal numbers, stocking rate and mean EPG counts among the flocks (Table 1), we sought a homogenous distribution of animals in the experimental groups from each flock.

The animals were treated with one of the following regimens: (1) albendazole: 3.4 mg/kg body weight (BW) of albendazole sulfoxide (sc); (2) closantel: 10 mg/kg BW of 10% sodium closantel orally after fasting for 12 h; (3) ivermectin: 0.2 mg/kg BW of 1% ivermectin (sc); (4) levamisole: 7.5 mg/kg BW of 7.5% levamisole hydrochloride (sc); (5) moxidectin: 0.2 mg/kg BW of 1% moxidectin (sc); and (6) control: untreated group. Ten to 14 days after treatment, another EPG count was performed and the feces obtained were submitted to culture for larval identification. The material was placed for 7 days in an incubator at 27 °C or for 10 days at room temperature. Then the infective larvae were morphologically classified according to helminth genera.

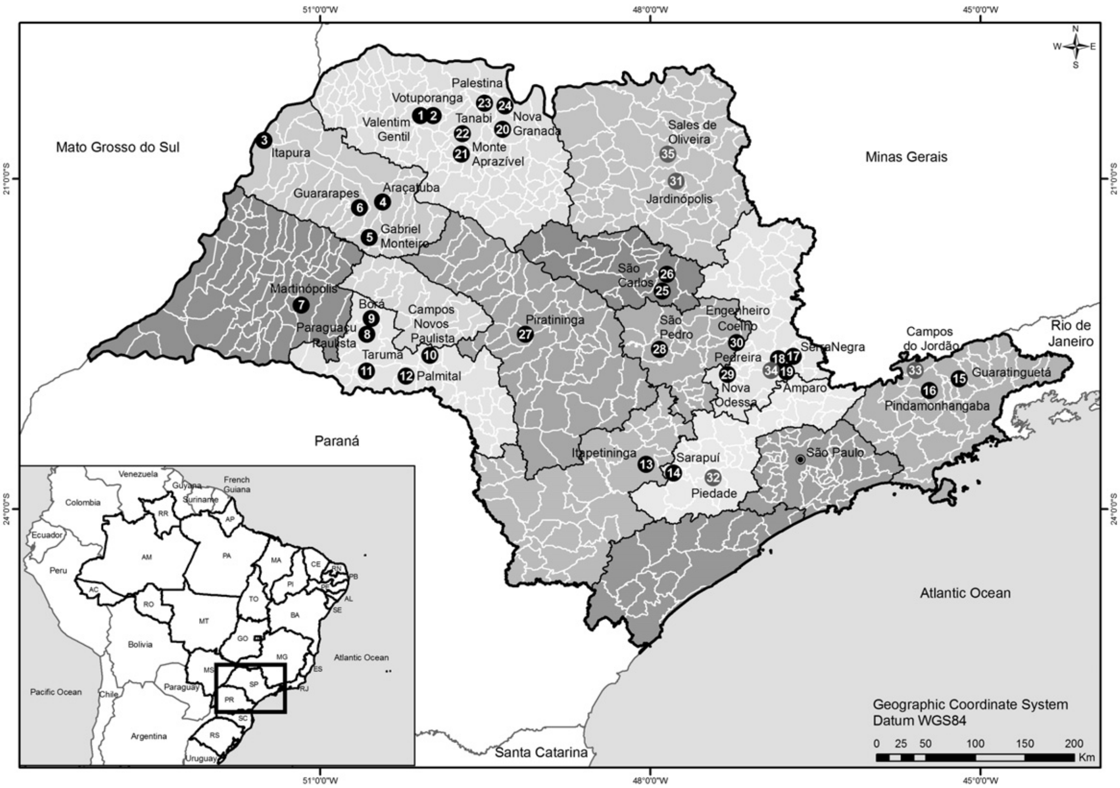

Fig. 1. Numbered circles indicate the 35 sheep farms in São Paulo state, Brazil. Black circles indicate farms evaluated by questionnaire and FECRT ($n = 30$); gray circles indicate farms evaluated only by questionnaire ($n = 5$).

2.3. Statistical analysis

The anthelmintic efficiency in the FECRT was calculated by the RESO 2.0 program (Wursthorn and Martin, 1990) using the formula $100 \times (1 - T/C)$, where T was EPG count 10–14 days after treatment and C was EPG count in the control group at the same time. Helminths were considered susceptible when the efficiency was above 90% and resistant when it was less than or equal to 90%. The helminth genus frequency after each anthelmintic treatment was compared to the frequency observed in the control group by the paired t-test with a significance level of 5%, calculated by the Minitab 13 program.

3. Results

3.1. Flock management practices

The farms' total areas ranged from 7.5 to 18,150 ha (median 105 ha) and the grazing pasture areas varied from 2 to 8470 ha (median 34 ha). The number of animals in each flock varied from 95 to 2028 (median of 301). The main breed was Santa Ines, followed by crossbred animals. Acquisition of new animals was not frequent (20.6%), and in most flocks (78.8%) the newly arrived animals were quarantined before being incorporated in the flock.

Rotational grazing was employed at 80% of the farms, and 82.9% of them followed a semi-intensive production system involving exclusive pasture in summer and feed supplementation or lamb confinement during winter. In 45.7% of farms, cattle and/or horses shared the same pastures with sheep.

In 64.7% of the flocks, anthelmintics were applied to only a few animals or categories, and in 70.6% of the flocks the dosage was based on visual estimation of the animal's weight. The FAMACHA system was used in 47.1% of flocks and clinical signs of parasitism were used as decision criteria for treatment in 41.2% of the flocks. Moreover, in half the flocks (50%), strategic deworming was also performed in females at the start of the breeding season and during the peri-partum period and in lambs at the weaning and growing/fattening stages. Few farms followed a fixed deworming schedule, every one (11.8% of the farms), two (5.9%), three (11.8%) or six (5.9%) months. Although anthelmintic rotation was performed when the drug had no effect on decreasing clinical signs at 44.1% of the farms, only 17.6% of the farmers performed the FECRT to make this decision. EPG counts were performed whenever necessary at 26.5% of the farms or with some fixed schedule at 20.6% of them.

Macrocyclic lactones were the main anthelmintics employed in the last three treatments (42.9, 50, and 54.2%), and most farmers (68.6%) did not use commercial or

Table 1
Number of sheep and stocking rate in each flock and mean (±SD) egg per gram (EPG) counts in animals (*N*) before and after the fecal egg count reduction test (FECRT).

Flock code	Number of sheep	Stocking rate[a]	*N*	Mean (±SD) EPG before FECRT	*N*	Mean (±SD) EPG after FECRT
1	350	29.2	60	1,960 ± 2,088	53	1,473 ± 3,408
2	1,215	9.1	48	512 ± 271	41	393 ± 759
3	484	28.5	111	1,087 ± 1,827	53	1,048 ± 1,396
4[b]	178	8.9	24	1,526 ± 2,111	25	2,910 ± 4,102
5	95	47.5	43	5,570 ± 8,263	43	1,849 ± 2,651
6	275	1.4	53	5,120 ± 5,695	47	2,410 ± 3,457
7	136	4.0	57	412 ± 744	57	229 ± 520
8	181	18.1	65	630 ± 953	62	954 ± 2,110
9	316	9.3	66	1,622 ± 2,372	64	1,262 ± 1920
10	286	2.0	62	2,538 ± 4,942	59	1,742 ± 3,066
11	183	22.9	64	1,235 ± 2,421	61	737 ± 1,109
12	488	24.4	67	1,924 ± 2,049	66	1,736 ± 3,743
13	168	2.8	83	2,611 ± 7,930	77	1,121 ± 3,118
14	1,240	26.1	50	2,675 ± 3,282	50	1,665 ± 2,516
15	301	3.3	60	1,047 ± 1,509	59	756 ± 1,619
16	1,997	13.8	60	3,908 ± 9,704	60	1,453 ± 1,913
17	153	34.8	117	1,410 ± 3,015	64	1,117 ± 1,643
18	226	–	66	1,586 ± 4,528	59	242 ± 488
19	1,200	58.3	84	363 ± 694	60	623 ± 2,270
20	2,028	27.9	70	4,747 ± 5,060	60	1,814 ± 2,368
21	742	14.5	66	1,527 ± 1,360	56	1,182 ± 1,480
22	716	18.5	84	4,022 ± 7,149	54	2,178 ± 3,164
23	160	22.0	56	2,876 ± 4,134	46	1,518 ± 2,363
24	777	14.0	85	8,591 ± 6,957	73	8,573 ± 8,843
25	217	36.2	59	773 ± 486	47	138 ± 245
26	2,000	–	10	630 ± 830	44	455 ± 1,124
27	280	–	35	1,407 ± 2,017	35	611 ± 1,146
28[c]	253	15.8	120	194 ± 437	27	228 ± 261
29	404	50.5	60	1,219 ± 4,009	59	397 ± 662
30	1,228	81.9	66	3,427 ± 3,073	56	701 ± 1,024
31[d]	–	–	98	116 ± 289	–	–
32[d]	214	5.9	21	876 ± 1,935	–	–
33[d]	750	0.1	31	1,380 ± 3,008	–	–
34[d]	244	3.1	22	514 ± 902	–	–
35[d]	–	–	10	5 ± 16	–	–

[a] Since a question about the stocking rate was not included in the questionnaire, it was calculated by the ratio between the number of animals and the pasture area of each farm, then differences among animal classes and lots may exist.
[b] FECRT only with albendazole compared to the control group.
[c] FECRT only with albendazole and moxidectin compared to the control group.
[d] Flocks not submitted to the FECRT.

self-formulated combinations of drugs or chemical groups for treatment of the animals.

3.2. Fecal egg count reduction test (FECRT)

Thirty flocks met the criteria for completion of the FECRT with at least one anthelmintic drug, for comparison against the control group (Fig. 1 and Table 1).

The frequencies of helminth genera observed after the FECRT in the six experimental groups are shown in Fig. 2. In all treatments, there was a predominance of *Haemonchus* sp. (average 75.8%), followed by *Trichostrongylus* sp. (19.1%) (Fig. 2). The other observed helminth genera were *Cooperia* sp. (2.5%), *Strongyloides* sp. (1.4%) and *Oesophagostomum* sp. (1.2%) (Fig. 2).

Compared to the control group, closantel treatment resulted in higher frequency ($P < 0.05$) of *Trichostrogylus* sp. (30.2% versus 19.5%) and *Cooperia* sp. (6.7% versus 1.8%) and in lower frequency ($P < 0.05$) of *Haemonchus* sp. (59.1% versus 74.4%), while ivermectin and moxidectin resulted in higher frequency ($P < 0.05$) of *Haemonchus* sp. (85.6% and 83.1% respectively, versus 74.4%) (Fig. 2).

The efficacy according to the FECRT of the five anthelmintics against all prevalent helminths is shown in Table 2 and Fig. 3 and against each helminth genera in Fig. 4.

Among the flocks, the efficacy values varied from 0 to 87% for albendazole, 2 to 95% for closantel, 0 to 86% for ivermectin, 0 to 100% for levamisole, and 0 to 99% for moxidectin (Table 2).

Anthelmintic resistance was considered to be efficiency values less than or equal to 90% in the FECRT. Helminths resistant to albendazole, closantel, ivermectin, levamisole and moxidectin were observed in 100, 92.9, 100, 53.6, and 96.6% of the flocks, respectively (Table 2). There was no farm where worms could be considered susceptible to more than two of the five evaluated anthelmintics and most of the flocks (53.6%) were resistant to all the five products (Table 2).

4. Discussion

This study reports the first systematic survey on the management practices adopted by sheep farmers in São

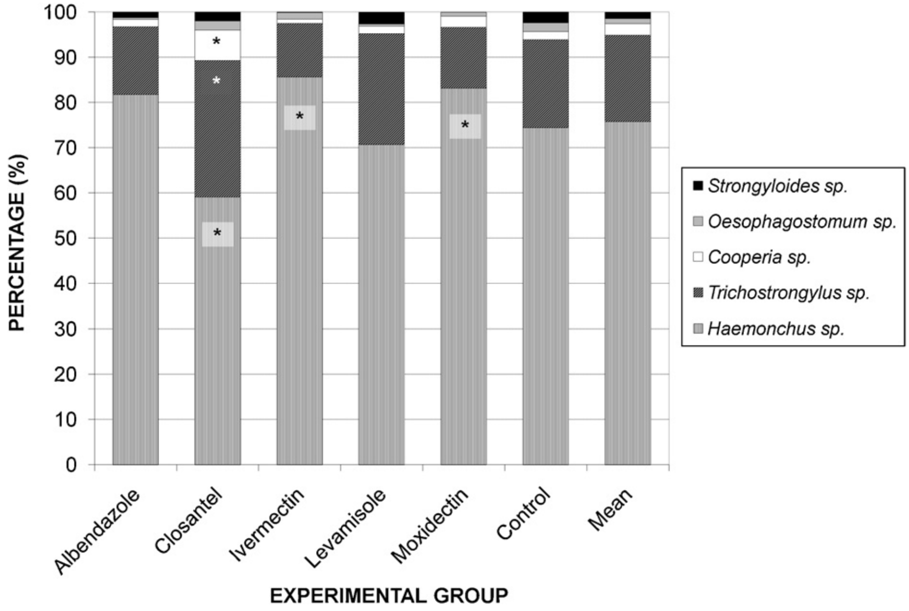

Fig. 2. Helminth genus frequency (%) after treatment with albendazole, closantel, ivermectin, levamisole, moxidectin, and control group and mean value among treatments. *Statistically different ($P < 0.05$) from the control group.

Table 2
Classification of helminths as susceptible or resistant after the fecal egg count reduction test with albendazole, closantel, ivermectin, levamisole, and moxidectin compared to the control group in sheep flocks and frequency of flocks with worms resistant to 0–5 anthelmintics (AH).

	Albendazole (%)	Closantel (%)	Ivermectin (%)	Levamisole (%)	Moxidectin (%)
Susceptible	0/30 (0%)	2/28 (7.1%)	0/28 (0%)	13/28 (46.4%)	1/29 (3.4%)
Resistant	30/30 (100%)	26/28 (92.9%)	28/28 (100%)	15/28 (53.6%)	28/29 (96.6%)
Resistance to anthelmintics (AH)					
0 AH	1 AH	2 AH	3 AH	4 AH	5 AH
0/28 (0%)	0/28 (0%)	0/28 (0%)	3/28 (10.7%)	10/28 (35.7%)	15/28 (53.6%)

Helminths in sheep flocks were classified as susceptible when anthelmintic efficacy was above 90%; and as resistant when anthelmintic efficacy was less than or equal to 90%.

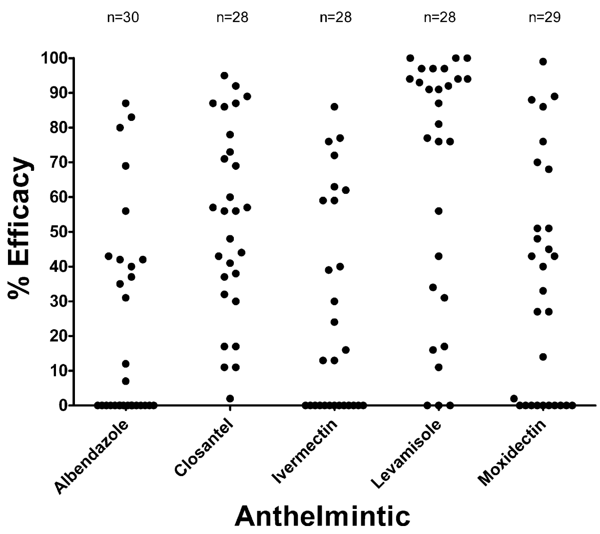

Fig. 3. Overall anthelmintic efficiency (%) of albendazole, closantel, ivermectin, levamisole and moxidectin compared to the control group in sheep flocks (n) after the fecal egg count reduction test.

Paulo state, Brazil. All told, 35 farms were surveyed and the efficacy of five anthelmintics was measured using the FECRT in the flocks at 30 of these farms. The differences in number of animals, stocking rate and mean EPG counts among the flocks reflects the situation of sheep production in São Paulo state and, as reported before, is in accordance with the established protocol for random selection of farms (Waghorn et al., 2006).

The effective size of flocks observed in São Paulo is larger than that reported in the states of Ceará (Melo et al., 2009) and Paraíba (Soares et al., 2009). The predominance of the Santa Ines breed reflects its good adaptation to tropical conditions and greater tolerance to nematode infections (Amarante et al., 2004). The use of crossbred animals (with Texel, Suffolk, Ile de France and Dorper) could improve meat production without increasing the sensitivity to gastrointestinal nematodes (Amarante et al., 2009).

Compared to surveys in other Brazilian states (Ceará and Paraíba), a similar frequency of semi-intensive farming was observed (Melo et al., 2009; Soares et al., 2009). However, the use of rotational grazing was higher than in the states of Ceará and Rio de Janeiro (Melo et al., 2009; Cruz et al.,

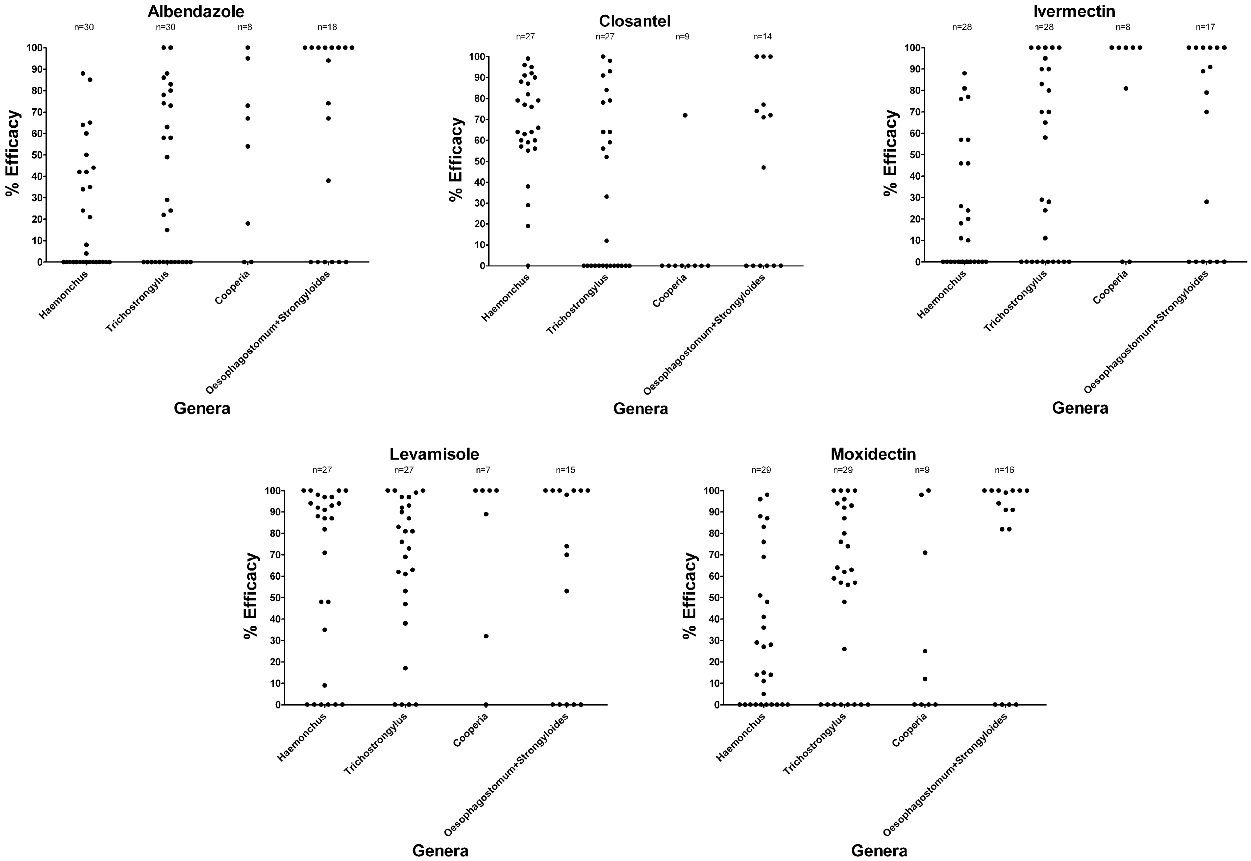

Fig. 4. Anthelmintic efficiency (%) of each helminth genus to albendazole, closantel, ivermectin, levamisole and moxidectin compared to the control group in sheep flocks (*n*) after the fecal egg count reduction test.

2010), but similar to Denmark (Maingi et al., 1996). In Slovakia (Čerňanská et al., 2008), the rate of sheep grazing with cattle was lower (10.6%) than observed here.

Similar rates of visual estimation of animal weight for treatment was observed in Denmark (84% for adults; Maingi et al., 1996), Mexico (97.4%; Torres-Acosta et al., 2003), and Slovakia (87.8%; Čerňanská et al., 2008). The rate of selective treatment based on clinical signs was similar to Mexico (44.8%; Torres-Acosta et al., 2003), but higher than in Rio de Janeiro state (18%; Cruz et al., 2010), which also used FAMACHA less frequently as a criterion for anthelmintic treatment decision (3%).

The predominant use of macrocyclic lactones was also reported in Rio de Janeiro (72%; Cruz et al., 2010), while in Ceará (Melo et al., 2009) the use of benzimidazole was more frequent. In Mexico (Torres-Acosta et al., 2003) and Slovakia (Čerňanská et al., 2008), benzimidazoles and macrocyclic lactones were also the most commonly used groups of anthelmintics.

Among the prevalent helminth genera, we observed the predominance of parasitism by *Haemonchus* sp. and *Trichostrongylus* sp. These results are consistent with several studies performed in Brazil (Ramos et al., 2002; Thomaz-Soccol et al., 2004; Rosalinski-Moraes et al., 2007; Melo et al., 2009; Sczesny-Moraes et al., 2010). Considering that these two parasites are very fecund (Coyne et al., 1991), it is not surprising for them to be the most prevalent in coprocultures from infected sheep.

In this study, all helminth genera were observed in the control group and the tested resistant groups, since they were obtained after all anthelmintic treatments (except *Strongyloides* sp. after moxidectin). The results observed for closantel should take into account that this anthelmintic is specifically targeted at *Haemonchus* sp. (Dash, 1986) and it is known not to be effective against many other helminth species. The higher frequency of *Haemonchus* sp. after ivermectin and moxidectin treatments may be due to the more frequent utilization of macrocyclic lactones, which can be selecting the most pathogenic helminth genera.

Anthelmintic resistance to GINs of small ruminants has being observed worldwide (Bartley et al., 2004). In this study, we found that multidrug resistance is widespread in São Paulo state, where 100% of the farms had worms resistant to albendazole and ivermectin, 96.6% to moxidectin, 92.9% to closantel, and 53.6% to levamisole. In a previous study involving ten farms in São Paulo state, resistance to oxfendazole and ivermectin was evident in 100 and 66.7% of the flocks, respectively, while susceptibility to levamisole was observed in 75% of the flocks (Amarante et al., 1992). In the southeastern region of Brazil, which includes São Paulo state, Farias et al. (1997) reported worms resistant to benzimidazole and levamisole in 96.4% and 91.3%

of the farms, respectively, while worms susceptible to ivermectin were present in 86% of the farms. Thus, this study indicates that parasite resistance is becoming more serious in São Paulo state. Also, the high levels of resistance reported here may reflect the situation of São Paulo as a new state in sheep farming, reflecting the acquisition of animals from different states, hence with the problem of anthelmintic resistance being imported from those states.

Resistant helminths to benzimidazol, closantel, levamisol, moxidectin and ivermectin were also reported in other Brazilian states: 74, 13, 30, not evaluated, and 77%, respectively (Ramos et al., 2002), and 75, 55.6, 44.4, 66.7, and 100%, respectively (Rosalinski-Moraes et al., 2007), in Santa Catarina; 88, 56, 38, 23.6, and 78.6%, respectively, in Paraná (Thomaz-Soccol et al., 2004); and 80, 80, 30, 33.3 and 80%, respectively, in Rio de Janeiro (Cruz et al., 2010).

In other South American countries, resistant helminths to benzimidazol, levamisole and ivermectin have been reported in Paraguay (73, 68, and 47%, respectively; Maciel et al., 1996), Uruguay (80, 71, and 1.2%, respectively; Nari et al., 1996), and Argentina (40, 22, and 6%, respectively; Eddi et al., 1996). In Malaysia, almost 50% of sheep farms presented worms resistant to benzimidazole, while resistance to levamisole, closantel and ivermectin was also observed (Chandrawathani et al., 1999). In New Zealand, resistance to albendazole, ivermectin and levamisole was evident in 41, 24, and 25% of flocks, respectively (Waghorn et al., 2006). In Spain, 18% of flocks were resistant to benzimidazole and 3% to macrocyclic lactones (Díez-Baños et al., 2008).

In the present study, resistance to both albendazole and ivermectin was observed in 89.3% of the flocks, and to albendazole and moxidectin in 82.8%. Resistance to ivermectin and moxidectin can also be mediated by β-tubulin, so the use of macrocyclic lactones may predispose to resistance to benzimidazole (Eng et al., 2006).

Although research and development in the veterinary parasitology field has improved considerably, with large research groups being formed using state-of-the-art facilities, in the past 25 years only one new class of anthelmintics, called amino-acetonitrile derivatives, with two classes of active ingredients – monepantel and derquantel – have been developed (Kaminsky et al., 2008). From the findings in this study, we consider that development of resistance to this product will likely start as soon the product reaches the market due to the lack of effective options and the increasing pressure for the use of drug combinations against highly resistant isolates. The best way to avoid this situation is to change from purely drug-based management to a more holistic approach for parasite control (Molento et al., 2011).

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