Multidrug resistance in *Haemonchus contortus* in sheep - can it be overcome?

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Abstract

Introduction: Gastrointestinal nematodes pose a threat to animal health and affect farmers by negatively impacting farm management. **Material and Methods:** The study was conducted on a sheep farm with suspected reductions in the efficacies of anthelmintics. Efficacy was determined using *in vivo* faecal egg count reduction, *in vitro* egg hatch (EHT) and larval development (LDT) tests. In the first phase, 60 sheep were equally split into six groups. Group 1 received the recommended dose of albendazole (ALB), group 2 received the same after fasting for 24 h, group 3 received the dose divided into two halves at 6 h intervals, group 4 received a double dose of ALB, and group 5 received the recommended dose of ivermectin (IVM). Group 6 served as a control. The second phase of the experiment had two groups: one treated with levamisole (LEV) and a control group. Faecal samples were collected from all sheep. **Results:** No reduction of egg output was observed in the groups treated with single, double, or divided doses of ALB, but one of 13.7–16.9% was noted in the fasting group. Efficacy in the IVM group ranged from 31.50 to 39.97%. The mean concentrations sufficient to prevent 50% of the eggs from hatching in the *in vitro* EHT and the mean concentrations in which the development of larvae to the L3 stage was inhibited by 50% in the LDT exceeded established thresholds for benzimidazoles and IVM. *Haemonchus contortus* was the only species identified after treatment. The LDT did not indicate the presence of resistance to LEV. All animals treated with LEV were negative for eggs 10 d after treatment. **Conclusion:** Resistance to ALB and IVM in *Haemonchus contortus* was confirmed. Alternative approaches to improve the efficacies of benzimidazole did not sufficiently increase the efficacy, but LEV was an efficient anthelmintic treatment.

Keywords: sheep, drug resistance, alternative treatment, *Haemonchus contortus*, levamisole.

Introduction

Infections with gastrointestinal nematodes (GINs) and the development of anthelmintic resistance (AR) remain constant threats to small-ruminant farms. The cost of helminth infections in dairy and meat breeds of sheep and goats in Europe is estimated at € 443 million annually. Another € 38 million per year is spent on gastrointestinal nematodes resistant to macrocyclic lactones (8). Cases of resistance to a single class of anthelmintics have been common in the past, but the occurrence of multidrug-resistant parasites is now increasingly reported in European countries. Multidrug-resistant populations of gastrointestinal nematodes on small-ruminant farms have been confirmed in central Europe – in Poland (19) and the Czech Republic (29). The occurrence of AR in goats was first reported in Romania (24), and multiple resistance to benzimidazoles and eprinomectin was observed in France (4). All these studies have been published in the last two years, and the dominant helminth species in most of them was *Haemonchus contortus*, which originally occurred in tropical and subtropical areas of south-eastern Asia, Africa, South America and Australia but is now commonly found on small-ruminant farms across Europe. The haematophagous *H. contortus* is the most pathogenic and fertile species of gastrointestinal nematodes, with adult females able

to produce 5,000–7,000 eggs per day (11). This ability allows this nematode to quickly contaminate large pasture areas and survive in hosts by constant reinfection. Applying effective control measures to stop the spread of haemonchosis is difficult under such conditions. Various alternative methods of treatment have been explored, *e.g.* using plant nutraceuticals against the parasites, but anthelmintics remain the basis of prophylaxis and the treatment of parasitic infections. The frequency of treatment and the application of the same class of anthelmintics for an extended period of time are among the main factors that contribute to the emergence of new cases of AR (28). Of the three classes of broad-spectrum anthelmintics, which are benzimidazoles (BZs), macrocyclic lactones (MLs) and cholinergic agonists (levamisole, LEV), only BZs and MLs are available for use in parasite control in sheep and goats in Slovakia. The first extensive studies on anthelmintic resistance performed in Slovakia in the 1990s (25) and mid-2000s (6) documented slow spreading of BZ resistance in Slovak sheep farms (up to 10% of farms were suspected to have resistant nematodes) (32). Later, because of the continued massive use of various BZ and IVM anthelmintics, the spreading of resistant populations of gastrointestinal nematodes among sheep farms increased (7, 13). Nowadays, the effectiveness of traditional anthelmintic treatment schemes is failing on isolated small-ruminant farms. Finding new approaches to improving the efficacies of the currently accessible anthelmintics is necessary due to the limited treatment options. In pursuit of better effects of the available anthelmintics, the main goal of this study was to modulate the availability of BZs in the sheep gut and to assess the *in vivo* efficacies of different schedules of treatment. Another goal was to identify resistant species of GINs *in vivo* and *in vitro* and to assess the effect of other classes of anthelmintics as potential treatments against resistant parasites on sheep and goat farms.

Material and Methods

Trial design. The study was conducted on a sheep farm located in the eastern part of Slovakia. Sheep of the Slovak dairy breed (n = 90) naturally infected with GINs were selected for the study. No animals selected for the study had been treated with any anthelmintics for two months prior to sampling.

Phase 1. Sixty sheep were split into six groups of 10 animals each. Group 1 (G1) was treated with the recommended dose of albendazole (ALB) of 5 mg/kg body weight (b.w.) (Albendavet; Divasa-Farmavic, Barcelona, Spain). Group 2 (G2) was treated with the same dose of ALB, but the animals were fasted for 24 h before treatment. In group 3 (G3), the recommended dose of ALB was divided in half and administered at 6h intervals. Group 4 (G4) was treated with a double dose of ALB at 10 mg/kg b.w. Group 5 (G5) was treated with ivermectin (IVM) (Ivomec; Merial, Lyon, France)

at a dose of 0.2 mg/kg b.w. Group 6 (G6) was not treated and served as a control. Faecal samples were individually collected directly from the rectum. All groups were resampled 10 d after treatment. The number of eggs per gram of faeces (EPG) was assessed by flotation using Sheather's sugar solution with a specific gravity of 1.28 based on a modified McMaster technique with a sensitivity of 50 EPG (9).

Phase 2. Another 30 sheep were divided into two groups of 15 animals each after the results from phase 1 were evaluated. One group was treated with the recommended dose of LEV (Interzan Gold Oral; Interchemie werken "De Adelaar" BV, Waalre, the Netherlands), and the other group served as a control. The subsequent faecal sampling and examination were the same as in phase one.

Faecal egg count reduction test (FECRT). The *in vivo* efficacies of the anthelmintics were determined using FECRTs based on the recommendations of the World Association for the Advancement of Veterinary Parasitology and Coles *et al.* (9, 10). Percent faecal egg count reduction (%FECR) was calculated using two formulae:

1. $\%FECR = (1/n)\ \sum(100\times(1-(T_{i2}/T_{i1})))$, where T_{i1} and T_{i2} are pre- and post-treatment EPGs, respectively, in host i from a total of n hosts (5);

2. $\%FECR = 100\times(1-(T_2/T_1))$, where T_1 and T_2 are the arithmetic means for the treated group before and after treatment, respectively, without a control group (16).

Egg hatch test (EHT). The EHTs were conducted as described by Coles *et al.* (10). Strongyle-type eggs from fresh pooled faecal samples were isolated by the washing of the faeces through a system of sieves (250, 100 and 25 μm). The suspension of eggs was subsequently added to 24-well plates and incubated at 27°C for 48 h in various concentrations (0.05, 0.1, 0.3, 0.5 and 1 μg/mL) of thiabendazole (TBZ; Merck, Darmstadt, Germany). Four replicates were used for each concentration. A well with 10 μL of dimethyl sulphoxide (Merck) instead of TBZ served as a control in each replicate. Incubation was ended after 48 h by adding 10 μL of an iodine solution (1 g of iodine and 2 g of potassium iodide in 100 mL of distilled water) to each well. The proportion of unhatched eggs and first-stage (L1) larvae was determined for each concentration using a Leica DM IL inverted microscope (Leica Microsystems, Wetzlar, Germany) at 40× magnification. The effective dose needed to prevent the hatching of 50% (ED_{50}) and 99% (ED_{99}) of the eggs was determined using a logistic regression model (12). An ED_{50} threshold of 0.05 μg/mL was used for TBZ (34).

Larval development test (LDT). The test was based on the methodology described by Hubert and Kerboeuf (15) and performed in a modified version proposed by Várady *et al.* (31). Helminth eggs were recovered from faecal samples using a set of three sieves of different mesh sizes (250, 100 and 50 μm).

The test was performed in 96-well microtitre plates which consisted of eight rows with 12 wells in each row. A suspension of approximately 80–100 eggs was incubated for 7 d at 28°C in a culture medium (yeast extract with Earle's balanced salt solution and physiological saline solution) in aquatic solutions of 12 concentrations for each of TBZ (0.0006–1.28 μg/mL; Merck), ivermectin aglycone (0.084–173.6 ng/mL; Merck) and levamisole (0.020–32 μg/mL; Merck). Four replicates were used for each concentration. Incubation was terminated after seven days by adding 10 μL of an iodine solution to each well. The distributions of eggs, L1, second-stage (L2) and third-stage (L3) larvae were assessed for each concentration. The results of the LDT were evaluated using the minimum inhibitory concentration (MIC) required to completely inhibit the development of L3 larvae. Minimum inhibitory concentration thresholds of 0.08 μg/mL, 21.6 ng/mL and 2.0 μg/mL were used for TBZ, IVM and LEV as described by Babják *et al.* (2), Dolinská *et al.* (13) and Taylor (27), respectively. Other parameters assessed were the lethal doses needed to kill 50% (LD_{50}) and 99% (LD_{99}) of the larvae, determined using a logistic regression model (12). A parasite population was evaluated as resistant when L3 larvae were present at concentrations equal to or higher than the MIC and when the LD_{99} was higher than the MIC threshold.

Morphological identification of L3 larvae. One hundred L3 larvae were identified according to morphological criteria before treatment and 10 d after treatment for each drug group, as described by van Wyk and Mayhew (30). Larvae at L3 stage were also isolated from the wells of the *in vitro* LDTs in "resistant" concentrations of 0.08–1.28 μg/mL TBZ, 21.6–173.6 ng/mL IVM and 2.0–32.0 μg/mL LEV and identified morphologically.

Results

Faecal egg count reduction test after phase 1. The results of the FECRTs are presented in Table 1. The mean EPG value in most of the experimental groups (G1, G3 and G4) was higher 10 d after treatment than on the day of treatment. The efficacy ranged from 31.50% to 39.97% in G5 (IVM) and from 13.70% to 16.90% in G2 (ALB, fasting) depending on the calculation method. In groups where post-treatment values of EPG exceeded pre-treatment results, the FECRT result was defined as 0%.

Phase 2. All 30 animals used in phase 2 were positive for strongyle-type eggs. Samples from these animals yielded EPG values ranging from 200 to 8,250. The mean EPG ± standard deviation for the treated and control groups were 2,233 ± 2,045 and 2,436 ± 1,239, respectively. All animals in the treated group were negative for GINs 10 d after the treatment (Table 1). Sheep in the control group remained positive, with a mean EPG of 2,297 ± 968.

Table 1. Results of the *in vivo* faecal egg count reduction test (FECRT): percent reduction 10 days after treatment in both phases of the trial

	Group (n = 10)/Treatment	Mean EPG ± SD	FECR (%)	
			Cabaret and Berrag formula (2004) (5)	Kochapakdee formula (1995) (16)
Phase 1	G1 Albendazole single dose			
	D0	370 ± 205	0	0
	D10	1,465 ± 1,482		
	G2 Albendazole fasting dose			
	D0	1,525 ± 1,212	16.90	13.70
	D10	1,317 ± 986		
	G3 Albendazole divided dose			
	D0	710 ± 326	0	0
	D10	1,400 ± 794		
	G4 Albendazole double dose			
	D0	605 ± 321	0	0
	D10	1,680 ± 1,803		
	G5 Ivermectin			
	D0	350 ± 345	39.97	31.50
	D10	240 ± 232		
Phase 2	Levasmisole			
	D0	2,233 ± 2,045	100	100
	D10	0		
	Control			
	D0	2,436 ± 1,239		
	D10	2,297 ± 968		

n – number of positive animals; EPG – eggs per gram; D0 – day of treatment; D10 – 10 days after treatment

Egg hatch test. Strongyle-type eggs hatched at all concentrations of TBZ. The mean hatching percentages at a TBZ threshold of 0.05 µg/mL and the highest concentration tested (1.0 µg/mL) were 96.00 ± 1.22 and 54.50 ± 3.20, respectively. The mean ED_{50} of 1.22 ± 0.08 considerably exceeded the threshold, indicating a high level of BZ resistance in the sheep. Mean hatching percentages from each concentration and value of ED_{50} are shown in Table 2.

Table 2. Mean hatching (in %) with standard deviation (SD) and mean of ED_{50} from the *in vitro* egg hatch test

TBZ concentration (µg/mL)	Mean hatching (%)	±SD
Control	96.25	0.43
0.05	96.00	1.22
0.1	91.25	4.22
0.3	84.00	1.73
0.5	71.50	4.55
1	54.50	3.22
Mean ED_{50} ± SD	1.22	0.08

TBZ – thiabendazole; ED_{50} – effective dose that inhibits egg hatching by 50%

Larval development test. The LDT results for all three classes of anthelmintics are summarised in Table 3. Larvae developed to L3 at all concentrations of TBZ. The mean percent of larval development to the infectious L3 stage at the MIC threshold of 0.08 µg/mL (2) was 56.31 ± 5.19%. The mean percentage of them showing development to the L3 stage at the highest TBZ concentration tested (1.28 µg/mL) was 34.18 ± 13.94%, and the mean LD_{50} was 0.37 ± 0.14 µg/mL. All data obtained indicated a high level of BZ resistance in the population. Similar results were obtained with IVM at the various concentrations. The mean percentage of larvae with development to the infectious L3 stage at a MIC of 21.6 ng/mL was 45.73 ± 6.32%. Development of the larvae stopped only at the highest concentration tested (173.6 ng/mL), and the mean LD_{50} was 28.87 ± 4.56 ng/mL. The *in vitro* data indicated a high level of IVM resistance, as in the case of BZ. The results agreed with the *in vivo* FECRT. Development to the L3 stage was inhibited in all replicates at LEV concentrations <2.0 µg/mL, and LD_{50} did not exceed the MIC threshold.

Morphological identification of L3 larvae. The proportions of L3 larvae before and after treatment are presented in Table 4. *Haemonchus contortus* was the dominant helminth species before the treatment of sheep and the only species identified after treatment. It was also the only species identified in the LDT wells at concentrations equal to or higher than the MIC threshold.

Discussion

The results of our study indicated the presence of multidrug-resistant *H. contortus* in the sheep herd examined. This study identified the second case of multidrug-resistant nematodes in Slovakia after a multidrug-resistant strain of *Teladorsagia circumcincta* was detected on a goat farm (3). Therefore, we assume that multidrug-resistant parasites on small-ruminant farms are becoming a serious problem in the country. Previously, numerous deaths of lambs had also been reported from the farm where we conducted our study, probably caused by multidrug-resistant *H. contortus*, against which the drugs administered were ineffective.

Table 3. Lethal dose (LD_{50}), minimum inhibitory concentration (MIC) and mean larval development ±SD at MIC thresholds for the three classes of anthelmintics obtained from the *in vitro* larval development tests

Drug	Larval development test		
	LD_{50}	MIC	L3 larvae (%) ± SD at MIC
Thiabendazole	0.37	n.a.	56.31 ± 5.19
Ivermectin	28.87	173.6	45.73 ± 6.32
Levamisole	0.10	1.0	0 ± 0

LD_{50} – lethal dose that kills 50% of the larvae; MIC – minimum inhibitory concentration; L3 – third stage larvae; n.a. – not applicable (no inhibition of development to L3 stage at the highest concentration of thiabendazole)

Table 4. Results of the morphological identification of third-stage larvae before and after treatment with albendazole (ALB), ivermectin (IVM) and levamisole (LEV)

Species	Before treatment (%)	After treatment (%)		
		ALB	IVM	LEV
Haemonchus contortus	77	100	100	—
Trichostrongylus spp.	13	—	—	—
Teladorsagia spp.	7	—	—	—
Oesophagostomum/Chabertia spp.	3	—	—	—

Nowadays, BZs and IVMs are the most widely used anthelmintics in Slovakia (7), and this will not change in the nearest future because they are still the only broad-spectrum drugs available on the market. The level of BZ and IVM resistance in the last survey conducted on sheep farms in Slovakia ranged from 20% to 30% (13). Our results indicated a large reduction in the efficacy of these drugs. In the present study, we determined anthelminthic efficacy using a well-established combination of *in vivo* and *in vitro* tests. The FECRT was assessed using formulae based on individual evaluations of EPG (5), which in previous studies were strongly correlated with *in vitro* tests and genotyping (2). A formula based on the arithmetic mean of treated groups before and after treatment (16) was also used. In the present study, we also applied the 24 h pre-treatment fasting schedule described by Hennessy *et al.* (14), which determined the effect of reduced feed intake on the prolonged availability of ALB in blood plasma in sheep. The interval two-step treatment schedule used for G3 was designed based on findings by Sanyal (26) to increase bioavailability and improve the efficacy of BZ when the recommended dose was administered in multiple doses. For comparison with these approaches, two other groups received the recommended dose of ALB for sheep and a double dose that should be applied in goats (33). As we could see in our study, EPG values did not decrease in the groups treated with the single (G1), double (G4) and divided (G3) doses of ALB, and the reduction was approximately 13–17% in the fasting group (G2). The FECRT results, as documented by the *in vitro* tests, indicated a high level of BZ resistance on the farm. Our results do not allow us to suggest that the applied treatment schedules would not be useful for increasing and improving the efficacy of the BZ anthelmintics, but the treatment schedules need to be well established before resistant populations of *H. contortus* can become highly dominant in Slovakia, as they were on this farm. Hennessy *et al.* (14) and Ali and Hennessy (1) confirmed that the efficacies of BZ and IVM against gastrointestinal nematodes in sheep increased by approximately 25–50% after applying these treatments. The level of resistance, however, was low or moderate in all cases, and the reduction of eggs after treatment was smaller in the tested population, unlike in our case when BZ (ALB) had no effect. In our study, the efficacy was the highest in the IVM-treated group. The reductions of EPG between 30% and 40% were well below the 90–95% range which is the threshold at which the drug is considered effective. The FECRT results indicated that different IVM treatment regimens would be more appropriate, but including more treatment groups in the experiment was not possible for logistical reasons. We also administered IVM subcutaneously, but changes in IVM pharmacokinetics have only been described for oral administration (1).

The results of the *in vitro* EHT were consistent with the *in vivo* FECRT. More than 90% mean hatching at the cut-off concentration of 0.05 μg/mL TBZ confirmed the presence of a highly BZ-resistant population on the farm. For comparison, mean hatching at the threshold concentration in a survey focusing on the occurrence of BZ resistance on goat farms in Slovakia ranged from 87% to 91% on the most-resistant farms (2), indicating that the efficacy of BZ against gastrointestinal nematodes was equally critical for both sheep and goats. The LDT results confirmed a critical situation with the efficacy of BZs and IVM on the farm. An LD_{99} higher than the MIC threshold was one criterion for evaluating a population as resistant, but this criterion was already exceeded in the BZ and IVM replicates at LD_{50}. The development of approximately 50% of the larvae to the L3 stage at threshold concentrations confirmed the presence of a high level of AR in *H. contortus* to both drugs. Monitoring larval development at threshold concentrations of 0.08 μg/mL for TBZ and 21.6 ng/mL for IVM is a reliable indicator of AR (2, 19, 24). Infectious L3 larvae were observed in our study at all TBZ concentrations tested, and development stopped only at the highest IVM concentration (173.6 ng/mL). Subsequent morphological identification of larvae in samples incubated with both drugs at concentrations equal to or higher than MIC indicated that all larvae were *H. contortus*. Levamisole was the only anthelmintic that could stop larval development before the MIC threshold of 2.0 μg/mL. The MIC for LEV should therefore be increased to 2.5 μg/mL in light of the presence of *H. contortus* (27). Following the LDT results, LEV was selected for the second phase of the *in vivo* experiment. Products based on LEV have not been available on the local market for a long time and had never been applied on the farm examined. Therefore, an efficacy of 100% in the treated group 10 days after its application was not surprising. The situation was similar on a goat farm in Slovakia, where a BZ- and IVM-resistant strain of *Teladorsagia circumcincta* was reported, but the efficacy of LEV was 94% (3). Levamisole was the only drug that did not produce AR in a study of 34 sheep herds in the Netherlands, which included five other anthelmintics: oxfendazole, moxidectin, IVM, monepantel and closantel (23). Also, LEV had efficacies of 98–100%, and fenbendazole and IVM had reduced efficacies, in sheep and goat dairy herds in Denmark (21). These data suggested that LEV-based products could be a solution to the ineffectiveness of BZ and IVM against gastrointestinal nematodes on small-ruminant farms. However, these drugs are not available in some countries, and their high prices can also be a barrier to their wider use. We believe that correct dosages as well as targeted selective treatments should be used to maintain the efficacy of LEV products; also, regular monitoring of the distribution of AR parasites will be necessary in the future. The threat

of the emergence of LEV resistance was confirmed in Australia, with efficacy levels from 73% to 96% (22). The occurrence of AR to LEV has also been reported in gastrointestinal nematodes of small ruminants in Europe – McMahon *et al.* (18) detected resistance to LEV in 14% of sheep herds in Northern Ireland and Mickiewicz *et al.* (19) reported the development of LEV resistance after one year of its intensive use in a goat herd in Poland. Other cases on small-ruminant farms have also been reported in Denmark (17) and France (20). Nevertheless, until now cases of LEV resistance in Europe have been rare. The main objective of cooperation between farmers and veterinarians should be to maintain the high efficacy of LEV for as long as possible using appropriate approaches to management and treatment.

In conclusion, we confirmed a case of multi-drug resistance in *H. contortus* on a sheep farm in Slovakia. We suppose that alternative methods of sheep treatment did not sufficiently increase the efficacy of the anthelmintics because resistance to BZ was strong. The one highly efficacious drug was LEV, and it was LEV and not the others probably because this class of anthelmintic drugs has not been used in the country for long. Levamisole is therefore probably the only effective protection against gastrointestinal nematodes on small-ruminant farms in Slovakia.

Conflict of Interests Statement: The authors declare there is no conflict of interests regarding the publication of this article.

Animal Rights Statement: All procedures were conducted following European Community guidelines (EU Directive 2010/63/EU). The experimental protocol was approved by the Ethical Committee of the Institute of Parasitology of the Slovak Academy of Sciences, adhering to national legislation in Slovakia (G.R. 377/2012; Law 39/2007) for the care and use of research animals. Permission to collect samples and to carry out the experiment was granted by the participating farmer.

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