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EFFECTIVENESS OF THE MOST OFTEN USED ANTHELMINTIC MOLECULES ON DIGESTIVE NEMATODES IN SHEEP

EFICACITATEA CELOR MAI DES UTILIZATE MOLECULE ANTIHELMINTICE ASUPRA NEMATOZILOR DIGESTIVI LA OI

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ABSTRACT | REZUMAT

Anthelmintic resistance (AR) is an increasing problem globally. Antiparasitic strategies that rely mainly on chemical molecules are increasingly threatened by the emergence of parasite strains resistant to numerous classes of anthelmintic substances. Benzimidazoles, imidazothiazoles, and macrocyclic lactones are the most frequent classes of broad-spectrum anthelmintics used against sheep nematodes. Multiple resistances are increasingly being reported for these compounds. In 2022, we performed a study in a herd from Cluj County, Romania, aiming to evaluate the efficacy of four anthelmintic synthetic molecules [albendazole (ABZ), levamisole (LEV), eprinomectin (EPR) and doramectin (DOR)]. Faecal samples were collected, before and 14 days after the treatment. The faecal egg count reduction test (FECRT) was used and coprocultures were performed for each group. Third-stage larvae (L3) were collected after 10 days from coproculture and identified using morphological and morphometrical keys. Before the treatment, we identified 5-6 strongyle genera/species in each group. *Haemonchus contortus* was the most resistant species. The population of digestive strongyles found in the herd under study showed resistance to all four used molecules; only the therapeutic combination consisting of levamisole and doramectin had efficacy > 95%. For *Strongyloides papillosus,* an efficacy of ≥ 95% was recorded following the administration of doramectin, eprinomectin and for the therapeutic combinations albendazole + doramectin and levamisole + doramectin.

Keywords: anthelmintic resistance, faecal egg reduction count, sheep

Rezistența la antihelmintice (AR) reprezintă o problemă la nivel global în creșterea rumegătoarelor. Strategiile antiparazitare care se bazează cu precădere pe molecule antihelmintice de sinteză sunt tot mai des amenințate de apariția unor linii de paraziți rezistenți la numeroase clase de substanțe antihelmintice. Cele mai frecvent utilizate antihelmintice cu spectru larg, împotriva nematodelor la ovine sunt: benzimidazolii, imidazotiazolii și lactonele macrociclice. Pentru acești compuși, rezistența multiplă este raportată din ce în ce mai des. Studiul nostru a fost realizat pe ovine dintr-un efectiv din județul Cluj, România, 2022. Testul de reducere a numărului de ouă din fecale (FECRT) a fost utilizat pentru a evalua eficacitatea a patru molecule sintetice antihelmintice: albendazol (ABZ), levamisol (LEV), eprinomectină (EPR) și doramectină (DOR). Au fost recoltate probe de fecale, înainte și după terapie și au fost efectuate coproculturi pentru fiecare grup. După 10 zile, L3 au fost colectate și identificate folosind chei morfologice și morfometrice. Pentru fiecare grup, am identificat 5-6 specii/genuri de strongili în probele colectate înainte de efectuarea terapiei. *Haemonchus contortus* a fost cea mai rezistentă specie. Populația de strongili digestivi prezentă la ovinele din efectivul luat în studiu s-a dovedit a fi rezistentă la toate cele patru molecule utilizate; doar combinația terapeutică constând în levamisol și doramectină a avut o eficacitate > 95%. Pentru *Strongyloides papillosus*, eficacitate ≥ 95% a fost înregistrată în urma administrării de doramectină, eprinomectină și pentru combinațiile terapeutice albendazol + doramectină și levamisol + doramectină.

Cuvinte cheie: rezistență la antihelmintice, testul de reducere a numărului de ouă din fecale, ovine

Nowadays there are three classes of broad-spectrum anthelmintics currently used against sheep nematodes: benzimidazoles, imidazothiazoles and macrocyclic lactones (6) and multiple resistance is increasingly reported (2, 10). The frequent use of the same anthelmintic molecules to control nematodes in livestock has led to high levels of anthelmintic resistance worldwide, in grazing ruminants (14, 18) and represents a serious threat to the livestock industry. Antiparasitic strategies that rely mainly on chemical molecules are increasingly threatened by the emergence of drug-resistant parasite strains that can survive the usual doses of numerous anthelmintic drugs (19). The resistance of parasites represents their hereditary abi-

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lity to tolerate a usual effective dose of a certain antiparasitic molecule. The nematode is considered to be resistant if it is not affected by exposure to the standard dose of anthelmintic and this characteristic is transmitted to the next generation of parasites (1). Administration of an effective antiparasitic drug will usually kill at least 95% of the gastrointestinal nematodes (18). The effectiveness of an anthelmintic drug depends on exposing the parasite to an appropriate dose, for sufficient time to get the targeted efficacy (18). Parasites that survive deworming, being genetically resistant, are those that will contaminate pastures with eggs and the resulting descendants will be carrying the resistance gene in turn (17). Three main groups of methods are used for the detection of anthelmintic resistance (AR): *in vivo* methods, the most intensively used is the faecal egg count reduction test (FECRT); *in vitro* methods - frequently used are the egg hatch test and the larval development test; molecular-based tests (6, 21). The World Association for Advance in Veterinary Parasitology (WAAVP), recommends FECRT as a standard AR detection method (5, 6) and sets the standards for conducting and interpreting the test (18). The main inconvenient of FECRT is the long period of time that is needed: 3-5 days for levamisole, 8 days for benzimidazoles, 14–17 days for macrocyclic lactones and 14 days if all the anthelmintic classes are used (6, 21). Taylor et al. (2002) mention a low sensitivity of FECRT to detect levels of resistance below 25% (21). The aims of this study were to evaluate the effectiveness of 4 anthelmintic synthetic molecules, from different classes, on sheep digestive nematodes and morphological identification of the digestive strongyles' species before and after therapy.

MATERIALS AND METHODS

Animals

The study was carried out in a herd of around 400 adult sheep, Țurcana breed, from Cluj County, Romania, during March 2022. The herd was grazed from March to November and during the winter the animals were housed in a wooden barn and fed with hay and concentrates (corn). Previously the animals were dewormed regularly in autumn and spring. The last treatment the animal received was albendazole 10 mg /kg PO (Gardal 10%, Intervet, Roumania) in the autumn of 2021.

Experimental protocol

Four molecules were tested: albendazole, levamisole, eprinomectin and doramectin to establish the effectiveness of the most frequently anthelmintic synthetic molecules used on sheep digestive nematodes. Animals were randomly selected, and divided into groups as follows: group 1 - albendazole (Gardal 10%), 7.5 mg/kg PO; group 2 - levamisole (Levaverm 10%), 10 mg/kg, PO; group 3 – doramectin (Dectomax), 0.2 mg/kg, IM; group 4 – eprinomectin (Eprecis), 0.2mg/kg, SC; group 5 - albendazole (Gardal 10%) + doramectin (Dectomax), 7.5 mg/kg + 0.2 mg/kg; group 6 - levamisole (Levaverm 10%) + doramectin (Dectomax), 10 mg/kg + 0.2 mg/kg. Each group consisted of 10 animals. Faecal samples were collected directly from the sheep rectum (10 samples/ treatment group) on days 0 and 14 of the experiment.

Sample analysis

The faecal samples were preserved at 4°C and examined within 48 h after collection at the Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, using Mc Master method to record the number of strongyle eggs per gram of faeces (EPG) (6).

Faecal egg count reduction test (FECRT)

The effectiveness of each antiparasitic product was calculated using FECRT, according to the following formula: FECRT%=(EPG 0 - EPG 14)/EPG 0 × 100, where EPG 0 represents the arithmetic mean of EPG from day 0 and EPG 14 represents the arithmetic mean of EPG from day 14

Anthelmintic resistance is suspected if the value of FECRT is less than 95% (3, 5).

Faecal culture

In order to morphologically identify 3rd stage larvae, samples with EPG values higher than 150 were selected to set up faecal cultures (16). For each group, 2 larval cultures were performed according to previously described protocols (9, 20). Faeces were placed in plastic cups, watered and covered with aluminium foil in which holes were made to maintain adequate oxygenation. The containers were maintained at 27 °C for 10 days (16, 23). During this time, the crops were sprayed with water, once every two days to maintain an optimal humidity for larval development (70-80%). Larvae were collected according to the following method: the containers with the faecal samples were filled with tap warm water and overturned in a Petri dish (16, 23). The Petri dish was filled with water, and then the samples were left at room temperature (22-23 °C) for 24 hours. During this time interval, the strongyle larvae migrated into the liquid from the Petri dish, which was collected with a pipette and put into 50 ml tubes (16). Afterward, the samples were kept at 4 °C until examination. Larvae identification was performed using the Olympus BX 61 microscope (4x, 10x, 20x and 40x objectives) and the Olympus Cell-F software. From each group 100 larvae were identified following the morphometric (length of the larva, tail and

tail filament) and morphological (the number of intestinal cells and their shape, the shape of the cephalic region of the larva and the terminal region) methods described by van Wyk, (2004), Zajac and Conboy (2012), and Knoll et al. (2021) (16, 23, 24).

Statistical analysis of data

The data were processed with MedCalc software. The arithmetic mean (± standard deviation) of EPG was calculated for each group.Then, ANOVA (one-way analysis of variance) was used to evaluate the statistical differences among groups. Differences were considered statistically significant if P-value was ≤ 0.05.

RESULTS AND DISCUSSIONS

Coprological examinations performed before any treatment revealed infections with digestive strongyles (100%) and *Strongyloides papillosus* (45%). The dynamics of the EPG during the experiment can be followed in Table 1.

Table 1

Average of faecal egg count (EPG before and after treatment) and statistical significance (p*)

Drug	Digestive strongyles Average ± SD		*S. papillosus* Average ± SD	
	Day 0	Day 14	Day 0	Day 14
ABZ	1105 (±781.54)	775 (±410.45)	40 (±73.78)	5 (±15.81)
	0.126*		0.079*	
LEV	1050 (±1186.49)	80 (±160.20)	75 (±76.46)	35 (±52.96)
	0.009*		0.222*	
DOR	1135 (±1111.81)	290 (±267.49)	100 (±81.64)	5 (±15.81)
	0.015*		0.005*	
EPR	535 (±366.70)	265 (±204.19)	50 (±47.14)	15 (±33.74)
	0.028*		0.066*	
ABZ+DOR	795 (±482.72)	155 (±160.64)	55 (±64.33)	0 (±0)
	0.004*		0.024*	
LEV+DOR	1005 (±1045.74)	5 (±33.74)	65 (±62.58)	0 (±0)
	0.003*		0.009*	

SD – standard deviation

Using the 6 therapeutic protocols, in the case of infections with digestive strongyles, significant differences were observed between the co-proeliminations from day 0 and day 14 for the molecules/combinations of anthelmintic molecules: levamisole ($p<0.01$), doramectin ($p<0.05$), eprinomectin ($p<0.05$), albendazole + doramectin ($p<0.01$), levami-sole + doramectin ($p<0.01$). No significant differences were recorded between coproeliminations, in the case of the use of albendazole. A significant reduction of EPG, after treatment, was observed for *S. papillosus* in the group treated with doramectin ($p<0.05$), albendazole+doramectin ($p<0.01$) and levamisole + doramectin ($p<0.01$).

The results of FECRT for each molecule/combination are presented in Table 2. A FECRT value ≥ 95% was observed for digestive strongyles only following the use of the therapeutic combination of levamisole + doramectin. According to our study, among the 4 products used alone, levamisole proved to be the most efficient in the case of digestive strongyles (92.38%). The use of levamisole in our country is quite low, one of the causes being its toxicity and possible side effects. The limit between the effective dose and the lethal dose is quite low, therefore, overdose can be easily reached (Menzies et al., 2010). In the case of *S. papillosus*, the FECRT value ≥ 95% was recorded for doramectin and also for the therapeutic combinations albendazole + doramectin and levamisole + doramectin. Resistance of *S. papillosus* was found for albendazole, levamisole and eprinomectin.

Table 2

Efficacy of molecules according to FECRT

Drug	Digestive strongyles Day 0/Day 14	*S. papillosus* Day 0/Day 14
ALB	29.86%	87.5%
LEV	92.38%	53.33%
DOR	74.44%	95%
EPR	50.46%	70%
ALB+DOR	80.50%	100%
LEV+DOR	99.50%	100%

The examination of larval cultures and the identification of species/genera of digestive strongyles found in pre- and post-therapeutic samples suggest resistance of some of them to various synthetic antiparasitic molecules. Before treatment, 5-6 parasitic species/genera were identified in each group, respectively: *H. contortus*, *T. circumcincta*, *Trichostrongylus spp*., *Cooperia* spp., *Oesophagostomum* spp., *C. ovina* and *S. papillosus*. The most prevalent species/genera identified were *H. contortus*, *T. circumcincta*, *Cooperia* spp., *Oesophagostomum* spp. and *S. papillosus* present in each group, and the lowest presence was found in the case of *C. ovina* (2 groups) and *Trichostrongylus* spp. (4 groups). At the second sampling, the variability of species/genera was much lower, only in the groups treated with albendazole and levamisole was revealed the presence of 6 and 5 different entities. *H. contortus* was the most prevalent species after the treatments, suggesting its resistance to all the molecules used in the experiment. An important increase in the percent of *H. contortus* L3 can be observed, within the D 14 larval cultures, for all the molecules used, with the highest percentage, respectively 100, being found in the case of the therapeutic combination levamisole + doramectin (Table 3).

Table 3

Identification of digestive strongyle species/genera pre- and post-therapeutic

Group	*H. contortus*		*Cooperia* spp.		*T. circumcincta*		*Trichostrongylus spp.*		*C. ovina*		*Oesophagostomum spp.*		*S. papillosus*	
	D 0 (%)	D 14 (%)	D 0 (%)	D 14 (%)	D 0 (%)	D 14 (%)	D 0 (%)	D 14 (%)	D 0 (%)	D 14 (%)	D 0 (%)	D 14 (%)	D 0 (%)	D 14 (%)
ALB	22	29	25	20	13	3	23	7	0	0	3	4	14	37
LEV	17	39	22	0	23	10	15	2	0	0	13	3	12	46
DOR	15	83	20	15	24	0	17	0	0	0	10	0	14	2
EPR	24	92	22	4	40	0	0	0	4	0	6	0	6	4
ALB+DOR	23	98	16	2	33	0	12	0	0	0	5	0	11	0
LEV+DOR	16	100	26	0	41	0	0	0	4	0	9	0	4	0

The genus *Cooperia* was the second most prevalent digestive strongyle found within this herd in D 14, the resistance being suspected for albendazole, doramectin, eprinomectin and albendazole + doramectin.

Widespread parasite resistance is determined by: frequent use of the same class of anthelmintics; preventive treatments for the entire herd, regardless of the condition of the animals; underdosing of anthelmintics; long-term use of a single antiparasitic drug. Intensive chemotherapy, often done without taking into account epidemiological aspects, is considered to be the main cause for the emergence of the phenomenon of resistance, which can be achieved through genetic mutations or can appear as an adaptive phenomenon (12). The ability of a parasite to resist usual doses of an antiparasitic molecule develops over a period of time of the order of years on a farm (17).

Benzimidazoles are one of the most used antiparasitic classes, being active against all nematodes and having an ovicidal effect (7). Efficacy varies widely depending on the degree of parasite resistance. Unlike the current study, where the efficacy of albendazole on sheep gastrointestinal nematodes was very low, a study conducted in the west side of the country, by Hora et al. (2014) using an albendazole-based product indicates an effectiveness of 97.03% (11). In the same part of Romania, Dărăbuș et al. (2012) establish an efficacy of 90.55% in sheep for albendazole sulfoxide (8). The anthelmintic resistance is genetically transmitted; a study conducted by Keyyu et al., (2002) demonstrated that after ten years of non-use, albendazole was only 59.4% effective (15).

Resistance can be detected for several classes of anthelmintics in the same flock. For example, in our study, the digestive strongyles were resistant to all anthelmintic molecules used (albendazole, levamisole, doramectin and eprinomectin). The phenomenon of multiple resistances has been reported in many countries of the world (4) to every anthelmintic class, causing an important loss for small-ruminant industries (13).

The results of the present study are comparable to other studies conducted in veterinary medicine on chemoresistance. A study carried out by Borgsteede et al., (2007), in the Netherlands, on a sheep farm with suspected resistance to avermectins after testing four anthelmintic molecules (doramectin, moxidectin albendazole and levamisole), obtained the following results: resistance for sheep treated with doramectin (15% efficacy) and albendazole (87% efficacy), levamisole and moxidectin having 100% and 99% efficacy respectively (2). Contrary to this study, the results obtained by us indicate 92.38% efficiency of levamisole on digestive strongyles thus resulting in a partial resistance. In Greece, a study conducted by Termatzidoua Sofia-Afroditi et al. (2017) revealed 98.8% efficacy of the same eprinomectin-based product (22) used in our study, a value much higher than we have found (52.67 %). Geurden et al. (2014) aimed to evaluate the efficacy of oral anthelmintics commonly used in Greece, France and Italy. They tested 4 molecules (moxidectin, ivermectin, levamisole and a benzimidazole), the highest efficacy being observed for the groups treated with moxidectin (99-100%) and ivermectin (98-100%). Larval identification, from the samples collected after treatment, indicated *Teladorsagia* spp. as the most common nematode larvae found, followed by *Haemonchus* spp. (10), unlike our study, where *H. contortus* was the most prevalent species after treatment, followed by the genus *Cooperia*.

CONCLUSIONS

The faecal egg reduction count test revealed the resistance of gastrointestinal strongyles to albendazole, levamisole, doramectin, eprinomectin and *Strongyloides papillosus* to albendazole and levamisole.

After the identification of the larvae resulting from the samples collected on day 0, polyspecific infections were found, 5 or 6 parasitic species/genera in each group (*H. contortus*, *T. circumcincta*, *Trichostrongylus* spp., *Cooperia* spp., *Oesophagostomum* spp., *C. ovina* and *S. papillosus*). The cultures made for the samples collected on day 14 p.t., revealed *H. contortus* as the most prevalent (6 groups), followed by *Cooperia* spp. (5 groups).

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