

IN VITRO EFFICACY OF ALBENDAZOLE AND DORAMECTIN ON SHEEP GASTROINTESTINAL STRONGYLES

EFICACITATEA *IN VITRO* A ALBENDAZOLULUI ȘI DORAMECTINEI ASUPRA STRONGILILOR GASTROINTESTINALI DE LA OVINE

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ABSTRACT | REZUMAT

Anthelmintic resistance (AR) in sheep digestive strongyles is a major challenge in controlling parasitic infections, significantly impacting animal health and causing considerable economic losses within the livestock sector. The most often used methods to determine the level of AR are *in vivo* test (faecal egg count reduction test), *in vitro* tests, and the molecular biology. *In vitro* methods for determining resistance are fast, can be used for multiple classes of anthelmintic, and detect low levels of resistance but require a large amount of work. In the spring of 2022, we carried out a study in two sheep herds from the Transylvania region, Romania, for detecting the effectiveness of two synthetic molecules, respectively: albendazole (ABZ) and doramectin (DOR), on sheep digestive strongyles. Two *in vitro* methods were used: the egg hatch test (EHT) for detecting resistance to benzimidazoles (BZ) and the larval development test (LDT) for identifying resistance across both classes of anthelmintics. The EHT revealed an efficacy for albendazole of 43.2% for the strongyles from the first flock and 60.8% for the second one. According to the results of LDT, the strongyle populations infecting the sheep in the first flock showed resistance to both tested molecules (albendazole: 43.2% and doramectin: 71.8%), while in the second flock, resistance was observed only to albendazole (61.6%), with the doramectin showing 100% efficacy.

Keywords: albendazole, digestive strongyles, doramectin, *in vitro* tests, sheep

Rezistența strongililor digestivi ai ovinelor la antihelmintice reprezintă o provocare majoră în controlul infecțiilor parazitare, având un impact semnificativ asupra sănătății animalelor și provocând pierderi economice considerabile în sectorul zootehnic. Cele mai des folosite metode pentru determinarea rezistenței sunt: testul *in vivo* (testul de reducere a numărului de ouă din fecale), testele *in vitro* și biologia moleculară. Metodele *in vitro* pentru determinarea rezistenței sunt rapide, pot fi utilizate pentru mai multe clase de antihelmintice, detectează niveluri scăzute de rezistență, dar necesită un volum mare de muncă. În primăvara anului 2022 s-a realizat un studiu în două efective de ovine din Transilvania, România, pentru depistarea eficacității a două molecule sintetice, respectiv albendazol și doramectină, asupra strongililor digestivi de la oi. Au fost utilizate două metode *in vitro*: testul de embrionare a ouălor pentru detectarea rezistenței la benzimidazoli și testul de dezvoltare larvară pentru identificarea rezistenței la ambele clase de antihelmintice. Testul de embrionare a ouălor a relevat o eficacitate pentru albendazol de 43,2 % pentru strongilii din primul efectiv și de 60,8% pentru al doilea. Conform rezultatelor testului de dezvoltare larvară, populațiile de strongili care au infectat oile din primul efectiv au prezentat rezistență pentru ambele molecule testate (albendazol: 43,2% și doramectină: 71,8 %), în timp ce la strongilii din al doilea efectiv, rezistența a fost observată doar la albendazol (61,6%), doramectina prezentând o eficacitate de 100%.

Cuvinte cheie: albendazol, strongili digestivi, doramectină, teste *in vitro*, oaie

Gastrointestinal nematodes (GIN) are widespread in grazing sheep, causing significant economic losses. The resistance of parasites to pharmaceutical products available on the market represents a current problem. Antiparasitic strategies that rely mainly on chemical molecules are increasingly threatened by the emergence of parasite-resistant lineages to numerous classes of anthelmintic substances (20). Parasite resistance is their hereditary ability to tolerate an 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 the anthelmintic, and this characteristic is transmitted to the next generation of parasites (1). Periodic deworming of small ruminants is the most frequently used method to control and avoid the spread of digestive nematodes (13, 21). The widespread use of synthetic molecules (5), combined with underdosing, lack of quarantine (2), and the absence of thorough monitoring to detect parasite presence, exacerbates the problem of AR. Anthelmintics have lost their efficacy, in some cases after only a few years of them being used (11, 12). The 'gold method' to determine the AR is the faecal egg count reduction test (FECRT), an *in vivo* method (13,

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14), which involves fieldwork and takes a long period of time, according to the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.): a minimum of 10 days for short-acting molecules like albendazole or levamisole and 28 days for long-acting macrocyclic lactone products. The most commonly used *in vitro* methods for detecting anthelmintic resistance are the egg hatch test (EHT) and the larval development test (LDT). The advantages of these tests are the short time needed before having the results (48 hours for EHA and 7 days for LDT), little fieldwork, inexpensive, easy to use (10), and high sensitivity to detect low levels of resistance (6,22). This study aimed to assess the effectiveness of two pharmaceutical anthelmintic molecules from different classes, very often used in Romania against gastrointestinal strongyles in sheep, using *in vitro* methods.

MATERIALS AND METHODS

The study was carried out in 2 sheep herds (H1 and H2) of adult male and female Țurcana breed sheep from Cluj County, Romania, during the spring of 2022. The last deworming for both herds was done using benzimidazole products (Gardal 10%, MSD and Albendakel 10%, KELA), 4 and 5 months, respectively, before our study. Individual samples were collected from 10 randomly selected animals in each flock. The samples were stored under anaerobic conditions until processing, within a maximum of 3 hours after collection. Pooled faecal samples weighing approx. 50 g were processed using the sieving techniques (sieves with mesh sizes of 200, 150, 100, and 36 μm) described by Coles et al. (2006) with some modifications. The eggs retained on the 36 μm sieve were transferred into centrifuge tubes. Supersaturated sugar solution (40%) was added to the tubes, which were then centrifuged at 2500 rpm for 5 minutes. The strongyle eggs were collected and washed with water. After being washed, the eggs were resuspended in deionised water, with the final concentration being approximately 100-150 eggs per 100 μl of suspension. Two tests were conducted for albendazole (EHT and LDT) and one for doramectin (LDT).

The egg hatch test (EHT)

This test is based on the ovicidal effect of benzimidazoles and the ability of resistant GIN eggs to embryonate and hatch when using anthelmintic doses

that would be lethal for sensitive strains (3). Thiabendazole is most commonly used in this test due to its high solubility, but other benzimidazoles can also be used for research purposes (22). In the present study, albendazole (Gardal 10%, MSD) was tested. A stock solution of albendazole (ABZ) was prepared using dimethyl sulfoxide (DMSO) as solvent (23).

In each well, 200 μl of egg suspension (approximately 100 eggs/100 μl), 10 μl of the diluted ABZ solution, and 790 μl of distilled water were added.

The final concentrations in the wells were 10, 5, 2.5, 1.25, 0.625, and 0.312 $\mu\text{g/ml}$, with 5 replicates for each concentration. Negative controls were also included for each concentration, where albendazole was replaced with DMSO. After 48 hours, during which the samples were incubated at 27°C in 24-well cell culture plates (Eppendorf, Nitech), two drops of Lugol's solution were added to each well, and 100 eggs and/or larvae were counted to determine the proportion of eggs that successfully embryonated and/or hatched.

The percentage of hatched eggs for each well was calculated using the following formula:

$$\frac{L1}{(L1 + \text{embryonated eggs} + \text{unembryonated eggs})} \times 100.$$

Larval development test (LDT)

This test is suitable for all antiparasitic classes (21).

The test principle is based on the larval ability to survive and develop in environments of various concentrations of anthelmintics (8).

The first step involved collecting eggs from faecal samples following the previously described method. ABZ dilution was done in the same manner as for the EHT. DOR was diluted in DMSO (7) due to its insolubility in water. The final concentrations in the wells were 86, 43, 21.5, 10.75, 5.375, and 2.687 ng/ml.

For each synthetic molecule, six concentrations were prepared with five replicates each. For the negative controls, the antiparasitics were replaced with DMSO. The plates were maintained at 27°C for 7 days (21), and then 100 parasitic elements from each well were counted to determine the proportion of L3 larvae.

Statistical analysis of data

One-way ANOVA analysis of variance was used to determine the statistical significance ($P < 0.05$). The results were analysed using MedCalc software. The arithmetic average and standard deviation (SD) of the efficacy were calculated for each group.

Table 1

Result of egg hatch test

Concentration ABZ		10 $\mu\text{g/ml}$	5 $\mu\text{g/ml}$	2.5 $\mu\text{g/ml}$	1.25 $\mu\text{g/ml}$	0.625 $\mu\text{g/ml}$	0.312 $\mu\text{g/ml}$
H1	Average $\pm$ SD	14 $\pm$ 6.70	36.6 $\pm$ 9.23	47 $\pm$ 16.47	56.8 $\pm$ 15.20	72 $\pm$ 8.94	74.4 $\pm$ 9.23
	Efficacy (%)	86	63.4	53	43.2	28	25.6
H2	Average $\pm$ SD	14.6 $\pm$ 6.42	20.8 $\pm$ 7.94	21.8 $\pm$ 8.07	39.2 $\pm$ 10.28	31.2 $\pm$ 8.70	57 $\pm$ 9.35
	Efficacy	85.4	79.2	78.2	60.8	68.8	43
p		0.9174	0.0369	0.0172	0.0431	0.0028	0.0027

SD – standard deviation; p – statistical significance

Table 2

Result for larval development test - albendazole

Concentration ABZ		10 µg/ml	5 µg/ml	2.5 µg/ml	1.25 µg/ml	0.625 µg/ml	0.312 µg/ml
H1	Average±SD	13.4±7.30	29.4±8.38	36.8±14.90	55.2±9.70	70.8±4.43	78.2±7.85
	Efficacy (%)	86.3	70.6	63.2	46.8	29.2	21.8
H2	Average±SD	14.6±6.34	22.4±9.39	22.6±3.84	38.4±11.28	32.8±19.69	58.8±13.02
	Efficacy (%)	85.4	77.6	77.4	61.6	67.2	41.2
p		0.8288	0.1907	0.0991	0.0433	0.0159	0.0105

SD – standard deviation; p - statistical significance

RESULTS AND DISCUSSIONS

Based on the results obtained, it can be concluded that, for H1, the efficacy of albendazole is considerably low at the discriminatory concentration of 1.25 µg/ml. While this concentration is expected to inhibit 99% (7) of egg embryonation in sensitive strains, the observed hatching rate was 56.8 (±15.20), indicating reduced effectiveness of the treatment. Even higher concentrations were ineffective, with the maximum efficacy reaching only 86% for the concentration of 10 µg/ml (Table 1). In H2 the hatching rate at the discriminatory concentration was 39.2% (±10.28). Similar to the first herd, the highest concentration tested achieved an efficacy of around 86%. Since an antiparasitic compound must eliminate at least 95% of parasitic formations to be considered effective, our results indicate a high level of resistance in both herds. Statistical comparisons between the two herds revealed significant differences in four of the six concentrations tested, with H1 exhibiting a higher resistance level.

LDT is considered the most versatile *in vitro* method for detecting anthelmintic resistance because it can be used to assess the efficacy of all classes of anthelmintic (8, 21). One limitation of this test is its longer duration (7 days) compared to EHT (48 hours). However, a key advantage is that it does not require eggs to be as fresh as those used in EHT. In our study the test was used for the same populations of digestive strongyles as the EHT. The resistance to albendazole was confirmed by LDT, the values of efficacy being 46.8% for the first flock and 61.6% for the second one (Table 2). For the two tests used, the results resembled each other; no statistical difference was observed between the first test and the second one regarding the efficacy of albendazole (p>0.05).

The second anthelmintic molecule tested belongs to the family of macrocyclic lactones, which were rela-

tively recently introduced and are already experiencing notable instances of resistance (9). The discriminatory concentration for macrocyclic lactones is considered to be 21.6 ng/ml (15, 10). As can be seen in Table 3, in H1 we found an efficacy of 71.8% which was much lower than the one from H2 (100%). The analysed data showed significant differences in all of the six concentrations tested.

In many countries worldwide, the phenomenon of multiple resistances has been reported. In several farms a certain degree of resistance to all available synthetic molecules has been detected (14). Based on the results presented earlier, we can conclude that the multiple resistance was present in the strongyle populations in one of the herds we studied. The strongyle populations in the first herd exhibit resistance to both albendazole and doramectin, while in the second herd they showed resistance only to albendazole, the doramectin having very good efficacy, as LDT indicated - 100%. In Romania, there are few studies on the anthelmintic resistance of GI strongyles in sheep. Magdaş et al. (2023) tested 4 synthetic molecules (albendazole, levamisole, eprinomectin, and doramectin) using an *in vivo* test (faecal egg count reduction test), showing resistance to all used anthelmintics (29.86% efficacy of albendazole and 74.44% for doramectin). The same test was used by Mateş et al. (2024) in two sheep herds from Romania; their results showed a higher level of efficacy 86.44% - 93.18% for albendazole and 94.73% - 97.67% for doramectin. In traditional grazing systems from our country, goats and other animal species are frequently found alongside sheep, with pastures being shared.

Most species of gastrointestinal strongyles in small ruminants are common, which is why we consider the mention by Potârniche et al. (2021) regarding the resistance of gastrointestinal strongyles in goats to albendazole and eprinomectin to be relevant.

Table 3

Result for larval development test – doramectin

Concentration DOR		86 µg/ml	43 µg/ml	21.5 µg/ml	10.75 µg/ml	5.375 µg/ml	2.687 µg/ml
H1	Average±SD	0	11.6±7.26	28.2±10.98	33.6±14.32	43.4±10.01	54.2±6.05
	Efficacy (%)	100	88.4	71.8	66.4	56.4	45.8
H2	Average±SD	0	0	0	4.2±3.70	17.6±6.02	28.4±7.43
	Efficacy (%)	100	100	100	95.8	82.4	71.6
p		0	0.0233	0.0045	0.0127	0.0115	0.0092

SD – standard deviation; p - statistical significance

Another example illustrating multiple resistance is provided by the study conducted by Cezar et al. (2010) in a herd of 5,000 sheep (Merino breed) from Brazil. Nine drugs were tested (levamisole, moxidectin, albendazole, ivermectin, nitroxynil, disophenol, trichlorfon, closantel, and a combination of ivermectin, levamisole, and albendazole); none of them had an efficacy higher than 95%. This indicates that the GIN present in that flock exhibit a certain degree of resistance to all the molecules tested. Dolinská et al. (2014) conducted a study in Slovakia on multiple sheep herds using *in vitro* tests and demonstrated that the resistance to albendazole was present in all 27 farms. Additionally, resistance to ivermectin was noted in 14 out of the 49 flocks where this molecule was tested. Kupčinskas et al. (2015) reported similar results regarding resistance after a study conducted in Lithuania using EHT and LDT. They identified resistance to albendazole in 100% of the herds tested, while resistance to ivermectin was observed in 13 out of the 21 farms examined. A large-scale study conducted over a period of 5 years in Spain (17) revealed AR rates of 27.3% for macrocyclic lactones, a value similar to that obtained by us in the first flock (28.2%). However, the resistance rate for albendazole (13.6%) was much lower than the value found in our study (38.4% - 56.8%). According to the studies presented and our own results, resistance of the sheep strongyles to benzimidazoles is more frequent and pronounced than resistance to macrocyclic lactones. This is likely due to the fact that the former were introduced to the market earlier, are inexpensive, easy to administer, have a broad anthelmintic spectrum, and are safe in terms of toxic dose. It is known that anthelmintic resistance is directly correlated with the popularity of anthelmintics often used in veterinary practice. Thus, the products based on albendazole are very often used for deworming sheep, a risk factor for the strongyles populations to develop resistance to the class of benzimidazoles.

CONCLUSIONS

The results of our study conducted on two populations of sheep digestive strongyles from two herds in Romania highlight the presence of resistance to albendazole in both populations, while resistance to doramectin was identified in one population. Continued surveillance of anthelmintic resistance in sheep strongyles in Romania is crucial. The veterinary practitioners must be informed of these findings and adjust deworming protocols to improve the control of gastrointestinal strongyles in small ruminants.

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