In Vivo and *In Vitro* Pharmacodynamics of Anthelmintic Medication Used in Sheep

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Abstract:

Our research aimed to evaluate the efficacy of anthelmintic treatments in sheep, as well as the resistance occurrence risk for the most commonly used substances. Anthelmintic medication efficacy was evaluated on 30 animals from a private farm, located in Sânmişiu Almaşului, Sălaj County. *In vivo* studies were performed by using Fecal Egg Count Reduction Test (FECRT) and testing an albendazole-based (ABZ) product. *In vitro*, we used Egg Hatch Assay (EHA) and Larval Development Assay (LDA) for albendazole (ABZ), mebendazole (MBZ), fenbendazole (FBZ), thiabendazole (TBZ) and ivermectin (IVM) (only for LDA). FECRT showed that intestinal nematodes developed resistance phenomena against the ABZ-based product, with an extensivity of 80% at seven days post therapy, an egg reduction percentage of 41.89% at seven days post-therapy and 43.9% at 14 days post-therapy. The *in vitro* EHA highlighted a superior efficacy of TBZ (egg hatch percentage at reference concentration being 51.21) compared to ABZ (71.89%), MBZ (84.46%) and FBZ (79.22%), with a minimum risk of anthelmintic resistance. The LDA test revealed the superior efficacy of FBZ (MIC 0.59 mcg/ml) and IVM (MIC 0.078 mcg/ml), with a minimal risk of inducing parasitic resistance. All *in vivo* and *in vitro* tests revealed a limited ABZ efficacy, recommending avoiding the therapy with this substance.

Keywords: Anthelmintic, EHA, FECRT, LDA, Pharmacodynamics, sheep, Romania

Introduction

For treating digestive strongyles in sheep, the more frequently used anthelmintics in Romania, are from the benzimidazole and macrocyclic lactones groups. The efficacy of these derivatives are seen by their ovicidal, larvicidal and adulticidal properties, depending on the pharmacologic class (Cristina, 1997; Riviere and Papich, 2009).

The parasitic resistance against anthelmintic medication is an important phenomenon, with real economic implications and having as result a lack of treatment solutions. Often veterinarians apply the antiparasitic treatments without knowing if there is efficacy or if the parasitic population already

developed resistance phenomena (Holm *et al.*, 2014). In this context, the aim of the present paper was to evaluate the efficacy of most commonly used anthelmintic treatments in sheep, as well as the evaluation of the resistance occurrence risk against these molecules.

Materials and methods

The present study was carried out on a population of 602 Tsurcana breed sheep, from which we selected 30 females (4.98%). The animals were located in a private farm in Sânmişiu Almaşului, Sălaj County, with ages ranging between nine and ten years. They were

maintained in traditional system, both on pasture and stabled. Five months prior testing, the sheep were dewormed using a fenbendazole based product.

Both *in vivo* and *in vitro* assessments were made. *In vivo* studies were performed by using Fecal Egg Count Reduction Test (FECRT) and evaluating an albendazole-based (ABZ) product used for routine antiparasitic treatments in this sheep population (Taylor *et al.*, 2002; Von Samson-Himmelstjerna *et al.*, 2009).

Faecal samples were obtained and evaluated before the antiparasitic treatment (BT) as well as at 7 and 14 days post-treatment (PT). We used McMaster method to quantify the total number of eggs per gram, parasitic intensity and extensivity BT and PT (Cerneia *et al.*, 2010a).

The total egg number per millilitre was determined applying the formula:

$$\text{Eggs/ml} = (1000 \times \text{no. eggs per } 50\mu\text{l}) / 50$$

Extensivity quantification was obtained by applying the following formula:

$$\text{E (\%)} = (\text{No. of positive samples} / \text{Total no. of samples}) \times 100$$

Parasitic intensity quantification was obtained by applying the following formula:

$$\text{I (EPG)} = \text{Total no. of EPG} / \text{No. of positive samples}, \text{ where EPG represents the number of eggs per gram of faeces}$$

The FCERT parasitic reduction percentage calculation was obtained by applying the following formula:

$$\text{FECRT} = [(\text{EPG BT} - \text{EPG PT}) / \text{EPG BT}] \times 100,$$

where EPG means eggs per gram of faeces, BT means before treatment, PT means post-treatment (Coles *et al.*, 1992; Von Samson-Himmelstjerna *et al.*, 2009).

In vitro, we used Egg Hatch Assay (EHA) and Larval Development Assay (LDA) for albendazole (ABZ), mebendazole (MBZ), fenbendazole (FBZ), thiabendazole (TBZ), and ivermectin (IVM)(only for LDA) (Von Samson-Himmelstjerna *et al.*, 2009).

EHA results interpretation was obtained by comparing them to those obtained at the reference concentration (0.15 mcg/ml), as *strongyle* populations in which eggs hatch in a percent of 50 or above are normally considered resistant (Coles, 1990; Taylor *et al.*, 2002).

For conducting *in vitro* bioassays, 2 ml well plates were used. The initial solutions consisted in serial dilutions of each substance, from 500

mcg to 0.98 mcg active ingredient/ml (a.i./ml). In each of the first 10 wells we placed 790 μ l of distilled water, 200 μ l of egg suspension and 10 μ l of each diluted active ingredient (a.i.). The final testing solutions contained from 5.0 to 0.0098 mcg a.i./ml. We used two controls, M₁ consisting of 200 μ l egg suspension and 800 μ l distilled water and M₂, consisting of 790 μ l distilled water, 200 μ l egg suspension and 10 μ l of HCl solution. In the statistical calculations we used the average obtained from the two controls. After 24 hours in the thermostat at 27°C, EHA assessment was carried out, by counting both the *unembryonated* and *embryonated* eggs as well as the hatched larvae. For the egg hatching percentage, the following formula was applied:

$$\text{Egg hatching \%} = [(\text{EE} + \text{L}_1) / (\text{UE} + \text{EE} + \text{L}_1)] \times 100, \text{ where } \text{L}_1 \text{ represents the number of first stage larvae, UE represents the number of } \textit{unembryonated} \text{ eggs and EE represents the number of } \textit{embryonated} \text{ eggs, (Coles } \textit{et al.}, 1992).$$

For Larval development assay (LDA), we obtained the minimum inhibitory concentrations (MIC) after keeping 14 days the exogenous parasitic forms in contact with the the diluted a.i. at a constant temperature of 27°C (Taylor *et al.*, 2002). Larval development percentage was calculated applying the formula:

$$\text{Larval development \%} = [(\text{L}_3 / (\text{UE} + \text{L}_3))] \times 100, \text{ where } \text{L}_3 \text{ represents the number of third stage larvae and UE stands for the number of } \textit{unembryonated} \text{ eggs.}$$

Determinations were performed within the Pharmacovigilance Laboratory of Cluj-Napoca Veterinary Medicine Faculty. All gathered data were centralised and statistically interpreted using Anthelmintic Resistance Program (ARP™), in order to obtain for each a.i., the following parameters:

- egg hatching percentage at the reference concentration (0.15 mcg / ml),
 - larval development percentage at the reference concentration (0.15 mcg / ml),
 - regression line and and its equation (Y parameters),
 - the mean concentration that inhibited hatching in 50% of the eggs (EC₅₀)
 - minimum inhibitory concentration (MIC)
- (Coles *et al.*, 1992; Von Samson-Himmelstjerna *et al.*, 2009; Cernea *et al.*, 2010b).

Table 1. The FCERT results obtained for ABZ before the treatment as well as 7 and 14 days PT

Sample	EPG BT	EPG 7 days PT	OPG 14 days PT
Total EPG	14800	8600	8300
Average	493.33	286.67	276.67
Standard Deviation	±1008.22	±268.76	±446.20
Extensivity (%)	66.66	80.00	70.00
Intensity (EPG)	740	358	395

Note: EPG= eggs per gram of faeces, BT = before the treatment, PT = post treatment

Table 2. Lower limit of the confidence interval and upper limit of the confidence interval for the tested strongyle population

Tested parameter	PT - 7 days	PT - 14 days
Percent of reduction (%)	41.89	43.91
LLCI 95%	-35	-48
ULCI 95%	75	79

Note: LLCI. 95%= lower limit of the confidence interval, ULCI95%= upper limit of the confidence interval

Results and discussions

The *in vivo* Faecal Egg Count Reduction Test.

By quantification of strongyle eggs we observed that, before treatment, the sheep presented an average of 493.33 EPG. At seven days PT, the level was considerably reduced, being 286.67 EPG. At 14 days PT we did not find significant changes compared to 7 days evaluation, average EPG count being 276.67 (Tab 1). Parasitic intensity before treatment was 740 EPG with a downward trend at seven days PT (358 EPG) followed by a slight increase at 14 days PT (395 EPG).

The extensivity dynamics for the strongyle population was 66.66% BT, and surprisingly reached the maximal value of 80% 7 days PT. Therefore the number of positive subjects, in the tested group, was bigger after treatment. At 14 days PT the extensivity level was 70% this value also being bigger than the one calculated BT. Extensivity and intensity dynamics highlights the reduced effects of ABZ against the tested adult digestive strongyles. This phenomenon is often seen and may be explained by the fact that ABZ acts mostly on the adult active parasitic forms and not on the ones in hypobiosis. After adult parasites being destroyed, the larvae in hypobiosis start a process of rapid transformation into adults and populate the intestinal lumen (Cernea *et al.*, 2010a; Coles, 2005). FECRT highlighted a parasitic reduction percentage of 41.89% at 7 days PT and 43.9% at 14 days PT.

Waller (1989) and Coles *et al.* (1992) consider that resistance phenomenon is present if the reduction percentage is less than 95% and if the 95% confidence interval is under 90% (lower limit of the confidence interval - LLCI 95%, upper limit of the confidence interval - ULCI 95%). If one of the two criteria exists, resistance phenomenon is suspected. According to the interpretation sequence for this test, the values are under the minimum accepted value of LLCI95%, indicating a reduced efficacy of ABZ against the tested strongyle population. The lower and upper limit of the 95% confidence interval were also under the maximum accepted limit of 90% (Tab. 2).

Therefore FECRT results presented the emergence of resistance phenomena for the tested parasitic population. Our results are in accordance with other studies connected to parasitic resistance, this phenomenon being seen in different parasitic species for all animal species and found worldwide (Cristina, 1997; Besier and Love, 2003; Kaplan, 2004; Manninen and Oksanen, 2010; Burgess *et al.*, 2012; Kaplan and Vidyashankar, 2012; Holm *et al.*, 2014).

The *in vitro* ABZ testing by EHA showed a hatching percentage of 80% at the reference concentration (0.15 mcg/ml), indicating reduced ovicidal effects. After statistical analysis of all hatching percentages obtained in all serial dilutions and control samples, the real hatching percentage at the reference concentration was actually 71.89% even so, way beyond the maximal accepted limit of 50%. EC₅₀ for ABZ was 1.26 mcg/

Table 3. *In vitro* EHA results obtained for albendazole and thiabendazole

Sample	A.i. conc. (mcg/ml)	ABZ				TBZ			
		No. of eggs	L1	Egg hatching %	Regression line equation	No. of eggs	L1	Egg hatching %	Regression line equation
1	5.0000	0	160	100.00	-23.41	120	60	33.33	-316.85
2	2.5000	40	80	66.67	25.72	140	100	41.67	-127.13
3	1.2500	60	100	62.50	50.28	80	140	63.64	-32.26
4	0.6250	40	300	88.24	62.56	140	60	30.00	15.17
5	0.3125	40	180	81.82	68.70	100	120	54.55	38.88
6	0.1563	20	80	80.00	71.77	100	80	44.44	50.74
7	0.0781	60	100	62.50	73.31	140	120	46.15	56.67
8	0.0391	240	300	55.56	74.07	80	100	55.56	59.63
9	0.0195	60	440	88.00	74.46	60	220	78.57	61.12
10	0.0098	40	180	81.82	74.65	60	140	70.00	61.86
HCl + H ₂ O	0.0000	30	90	75.00	74.84	60	170	73.91	62.60
Total		630	2010			1080	1310		
	Parameters		Hatching % ref.	EC ₅₀ (mcg/ ml)		Parameters		Hatching % ref.	EC ₅₀ (mcg/ml)
	a	-19.65	71.8900	1.2600		a	-75.89	51.2100	0.1600
	b	74.84				b	62.6		

Note: A.i. conc. = active ingredient concentration, EC₅₀ = mean concentrations that inhibited hatching in 50% of the eggs, Hatching % ref. = Hatching percentage at reference concentration of 0.15 mcg/ml

ml and the regression line had a negative tendency, Ymax parameter having a negative value of -23.41. These data state the fact that the tested population of digestive nematodes have the tendency to develop adaptative phenomena against ABZ (Tab. 3).

For TBZ, EHA results were superior to those obtained in ABZ and MBZ. At the reference concentration, the real hatching percentage was 51.21%, superior to the minimal accepted value. EC₅₀ presented a value of 0.16 mcg/ml, indicating the fact that TBZ is active against strongyle eggs, having an ovicidal effect at very low concentrations. The good TBZ efficacy is also confirmed by the calculation of Ymax parameter which showed a negative trend for the regression line (Ymax = -316.85) (Tab.3).

When testing MBZ, the real hatching percentage at the reference concentration was determined to be 84.46% (Tab. 4). EC₅₀ for was - 0.54 mcg/ml indicating that this substance has a really low efficacy, even at the lowest tested dilution (5 mcg/ml). The regression line equation had the Ymax parameter 324.63. Statistically, the presented data is interpreted by the fact that in order to obtain positive values for EC₅₀ we should have worked with concentrations higher than 5 mcg/ml, concentrations that cannot be justified

and applied as treatments, making this substance not usable for the tested digestive nematode population.

We included FBZ for the *in vitro* tests because the sheep population was treated with a FBZ based product five months prior the ABZ treatments. EHA results for FBZ showed a hatching percentage of 79.22 at reference concentration indicating a reduced efficacy against the tested parasite population (Tab. 4). The calculated EC₅₀ was -0.69 mcg/ml and the regression line had a positive tendency (Ymax = 246.35), indicating a high risk of resistance against this substance. The comparative analysis of the tested parameters in EHA for the four benzimidazoles revealed the superior efficacy of TBZ and a reduced efficacy for ABZ with a very high risk of resistance phenomena. For MBZ and FBZ analysed data showed a very low efficacy, their use not being recommended anymore for the tested population.

The LDA *in vitro* tests have the advantage of having a prolonged contact (10-14 days) between the anthelmintic substance and the exogenous parasitic forms. For ABZ testing, the presence of L3 larvae was observed starting with the concentration 2.5 mcg/ml, therefore we can state that prolonging the contact time between this substance and the parasites did not have

Table 4. *In vitro* EHA results obtained for mebendazole and fenbendazole

Sample	A.i. conc. (mcg/ml)	MBZ				FBZ			
		No. of eggs	L1	Egg hatching %	Regression line equation	No. of eggs	L1	Egg hatching %	Regression line equation
1	5.0000	20	160	88.89	324.63	40	60	60.00	246.35
2	2.5000	60	140	70.00	200.83	40	100	71.43	160.20
3	1.2500	20	120	85.71	138.93	40	60	60.00	117.13
4	0.6250	40	180	81.82	107.98	20	160	88.89	95.59
5	0.3125	20	80	80.00	92.51	20	60	75.00	84.82
6	0.1563	40	180	81.82	84.77	40	240	85.71	79.44
7	0.0781	40	400	90.91	80.90	20	80	80.00	76.74
8	0.0391	60	100	62.50	78.97	60	120	66.67	75.40
9	0.0195	20	160	88.89	78.00	20	60	75.00	74.72
10	0.0098	60	160	72.73	77.52	100	260	72.22	74.39
HCl + H ₂ O	0.0000	40	90	69.23	77.03	30	60	66.67	74.05
Total		420	1770			430	1260		
Parameters		Hatching % ref.		EC ₅₀ (mcg/ml)		Parameters		Hatching % ref.	
a		49.52		84.46		a		34.46	
b		77.03		-0.5400		b		79.2200	
								-0.6900	
								74.05	

Note: A.i. conc. = active ingredient concentration, EC₅₀ = mean concentrations that inhibited hatching in 50% of the eggs, Hatching % ref. = Hatching percentage at reference concentration of 0.15 mcg/ml

Table 5. *In vitro* LDA results obtained for albendazole and mebendazole

A.i. conc. (mcg/ml)	Sample no.	ABZ				MBZ			
		Eggs + L1 + L2	L3	Larval development %	Regression line equation	Eggs + L1 + L2	L3	Larval development %	Regression line equation
5.0000	1	60	0	0.00	-50.29	120	20	14.29	-50.09
2.5000	2	20	20	50.00	-14.32	100	0	0.00	-14.67
1.2500	3	20	0	0.00	3.67	80	20	20.00	3.05
0.6250	4	40	20	33.33	12.67	100	80	44.44	11.90
0.3125	5	100	0	0.00	17.16	120	80	40.00	16.33
0.1563	6	20	0	0.00	19.41	40	20	33.33	18.55
0.0781	7	100	60	37.50	20.54	20	0	0.00	19.65
0.0391	8	240	60	20.00	21.10	100	20	16.67	20.21
0.0195	9	120	20	14.29	21.38	80	20	20.00	20.48
0.0098	10	60	40	40.00	21.52	140	20	12.50	20.62
0.0000	H ₂ O +HCl	110	20	15.38	21.66	30	10	25.00	20.76
Total		890	240			930	290		
		MIC - mcg/ml		Parameters		MIC - mcg/ml		Parameters	
		1.3300		a -14.39		1.1100		a -14.17	
				b 21.66				b 20.76	

a significant effect on increasing the substance efficacy. The minimum inhibitory concentration (MIC) determined by statistical calculations was 1.33 mcg/ml, the regression line tendency being slightly negative (Ymax = -50.29) (Tab. 5). The limited ABZ efficacy could be explained by the *in vivo* ABZ pharmacokinetics. After being administered, ABZ enters the enterohepatic circuit, being transformed in active metabolites,

transformation that is not possible when testing the substance *in vitro* (Riviere and Papich, 2009).

For MBZ, LDA test showed a higher efficacy compared to the one obtained in EHA. even though the first L3 larvae were observed at 5 mcg/ml concentration, their percentage was low (14.29%). MIC was 1.11 mcg/ml, better than the one of ABZ (1.33mcg/ml). The superior efficacy of MBZ in LDA test was also observed when calculating the

Table 6. *In vitro* LDA results obtained for thiabendazole and febendazole

A.i. conc. (mcg/ ml)	Sample no.	TBZ				FBZ			
		Eggs + L1 + L2	L3	Larval develop- ment %	Regression line equation	Eggs + L1 + L2	L3	Larval develop- ment %	Regression line equation
5.0000	1	20	0	0.00	64.62	40	0	0.00	-183.64
2.5000	2	100	20	16.67	46.12	60	20	25.00	-78.07
1.2500	3	20	0	0.00	36.87	60	0	0.00	-25.28
0.6250	4	60	40	40.00	32.25	40	20	33.33	1.12
0.3125	5	80	20	20.00	29.93	20	20	50.00	14.31
0.1563	6	120	20	14.29	28.78	60	40	40.00	20.91
0.0781	7	80	40	33.33	28.20	20	0	0.00	24.21
0.0391	8	80	20	20.00	27.91	60	20	25.00	25.86
0.0195	9	60	40	40.00	27.76	80	20	20.00	26.69
0.0098	10	100	40	28.57	27.69	40	20	33.33	27.10
0.0000	H ₂ O +HCl	150	50	25.00	27.62	70	30	30.00	27.51
Total		870	290			550	190		
		Parameters				Parameters			
		MIC - mcg/ ml		a	7.4	MIC - mcg/ml		a	-42.23
		-3.3900		b	27.62	0.5900		b	27.51

regression line equation and the Ymax parameter (-50.09) (Tab. 5)

Compared to EHA results, TBZ in LDA test presented a limited efficacy against larval development, the maximal percentage of larval development (40%) being observed at 0.625 mcg/ml and 0.0195 mcg/ml.

For the LDA, the presence of infecting larvae was observed for all tested TBZ dilutions, excepting 5 mcg/ml and 1.25 mcg/ml. MIC had a negative value (-3.39 mcg/ml) and the regression line equation had a positive trend, having a Ymax parameter of 64.62. For this substance, in order to be able to obtain positive values for MIC, we should have worked with concentrations higher than 5 mcg/ml (Tab. 6). Due to this low efficacy, nowadays TBZ is rarely used in veterinary medicine.

In vitro LDA testing for FBZ showed surprising results, superior to those obtained in EHA. MIC was 0.59 mcg/ml, the regression line tendency being negative (Ymax = -183.64) (Tab. 6). When comparing the results of the two *in vitro* tests for FBZ we observed a better efficacy for this substance if there is a prolonged contact time and a better larvicidal capacity compared to ovicidal action. As practical applicability of these results, we would recommend that FBZ to be used only if

there is the possibility of repeating the treatment at 7-10-14 days interval (Kaplan, 2004).

The last tested larvicidal substance tested *in vitro* by using LDA was IVM. For this substance L₃ larvae were observed in almost all of tested serial dilutions, except 0.15 mcg/ml and 0.078 mcg/ml. MIC was equal to the one obtained in FBZ (MIC = 0.59mcg/ml), the regression line showing a negative tendency (Ymax= -156.81) and a low risk of inducing resistance phenomena (Tab. 7). When comparing all results obtained in LDA, we observed the high efficacy of FBZ and IVM. The minimal risk of inducing resistance phenomena was seen in FBZ followed by IVM, but we must not forget that for ABZ the lack of active metabolites for *in vitro* testing can offer different results.

The applied *in vitro* and *in vivo* assays for ABZ showed a clear correlation between FCERT and the LDA/EHA results. All performed tests showed a low efficacy of ABZ, reason for which we recommend that, from now on, this sheep population to be treated with products based on IVM or FBZ if, for the last one, the treatment can be repeated in 10-14 days (Manninen and Oksanen, 2010).

Table 7. *In vitro* LDA results obtained for ivermectin

A.i. conc. (mcg/ml)	Sample no.	IVM			
		Eggs + L1 + L2	L3	Larval development %	Regression line equation
5.0000	1	20	40	66.67	-156.81
2.5000	2	40	20	33.33	-65.01
1.2500	3	20	20	50.00	-19.11
0.6250	4	100	20	16.67	3.84
0.3125	5	0	20	100.00	15.32
0.1563	6	40	0	0.00	21.05
0.0781	7	20	0	0.00	23.92
0.0391	8	160	60	27.27	25.35
0.0195	9	40	20	33.33	26.07
0.0098	10	120	20	14.29	26.43
0.0000	H2O +HCl	30	20	40.00	26.79
Total		590	240		
		MIC - mcg/ml		Parameters	
		0.5900		a	-36.72
				b	26.79

Conclusions

The *in vivo* FECRT revealed the fact that the tested strongyle population is resistant against ABZ based medication.

In vitro EHA showed a superior efficacy of TBZ and reduced efficacy for ABZ, this last substance having also a maximal risk of inducing resistance phenomena. For MBZ and FBZ a low efficacy was observed;

LDA showed a superior efficacy for FBZ proving that, in this case, a longer contact time can have better effects. The lower risk of inducing resistance phenomena was observed in FBZ followed by IVM.

The applied *in vivo* and *in vitro* assays showed a clear correlation between the results, all indicating a reduced efficacy of ABZ.

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