***In Vitro* Study of Benzimidazole Derivatives, Tetrahydropyrimidines and Macrocyclic Lactones Therapeutic Efficacy in Dog Hookworms**

Laura CĂȚANĂ^{1*}, Raul CĂȚANĂ¹, Roxana CORA¹, Ștefan RĂILEANU², Mihai CERNEA¹

¹*University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Faculty of Veterinary Medicine, 400372, Calea Mănăștur, No. 3-5, Cluj-Napoca, (România)*

²*Danube Delta National Institute for Research and Development, Babadag Street, no. 165, PO 820112, Tulcea City, (Romania)*

*corresponding author: laura.catana@usamvcluj.ro

Bulletin UASVM Veterinary Medicine 75(2)/2018

Print ISSN 1843-5270; Electronic ISSN 1843-5378

doi:10.15835/buasvmcn-vm:2018.0019

Abstract:

The study was conducted using faecal samples from 62 dogs. We tested the ovicidal and larvicidal effects of albendazole (ABZ), mebendazole (MBZ), fenbendazole (FBZ) and *flubendazole* (FLU) by Egg hatch assay (EHA) and larval development assay (LDA). For pyrantel (PYR) and selamectin (SEL) we tested the larvicidal effects by LDA. In all *in vitro* tests, benzimidazoles efficacy was low, with a high risk of inducing resistance phenomena. In EHA more than 50% of the *hookworm* eggs hatched, revealing a low efficacy of all tested benzimidazoles. The regression line was positive for all benzimidazoles, FBZ having the smallest value of the Y parameter (62.62), and lower risk of resistance. When testing the larvicidal effects, a superior efficacy of benzimidazoles was observed. The lowest MIC was for MBZ (0.8672 μg/ml). ABZ had a very poor effect (8.46750 μg/ml). The Y parameter showed a lower risk of inducing resistance for MBZ (Y= -64.14) and FBZ (Y= -27.89). Pyrantel and Selamectin were very effective, presenting also a very low risk of inducing resistance phenomena. For PYR and SEL, MIC was 0.2131 μg/ml and 2.7921 μg/ml, respectively. The Y parameter was -448.37 for PYR and -62.74 for SEL, with minimal risk of inducing the adaptive phenomena.

Keywords: *Ancylostomatidae*, benzimidazoles, dog hookworms, EHA, LDA, Pyrantel, Selamectin

Introduction

Ancylostomatids are some of the most important parasites affecting dogs worldwide, in terms of diffusion and risk for animal and human health (Traversa, 2012; Wright, 2009).

They are also pathogenic in humans, causing cutaneous larva migrans (CLM) or creeping eruption and eosinophilic enteritis (Brenner and Patel, 2003; Croese *et al.*, 1994).

Infective stages are either ingested by the definitive host as free-living third-stage larvae, L3, lactogenically, or via paratenic hosts. However, the L3 also are able to penetrate percutaneously after complex neurohormonal actions that are triggered

by skin conditions (Epe, 2009). Most pet owners do not follow the manufacturers and doctors recommendations, deworming at irregular and consequently ineffective intervals (Cerneza *et al.*, 2010; Matos *et al.*, 2015). In the past few years the scientific *community's attention* has been attracted by feline and canine extra-intestinal parasitic nematodes. This change led to the misconception that intestinal worms of cats and dogs do not deserve a high-standard level of attention anymore (Traversa, 2012; Robertson and Thompson, 2002). These problems occur worldwide, both in rural and urban areas. Drug resistance, defined by WHO as the ability of parasites in a population

to survive drug treatment, which is generally effective against the same species at the same stage of infestation at an identical dosage level (World Health Organization, 2011) has become commonly reported. One of the biggest concern is the resistance against benzimidazole compounds (Cernea *et al.*, 2010), associated, for example, to single nucleotide polymorphisms in the β -tubulin isotype 1 gene (VianaFurtado *et al.*, 2014).

The present paper aims was to evaluate the efficacy of selected members of benzimidazole, tetrahydropyrimidine and macrocyclic lactone anthelmintics groups against canine hookworms.

Materials and methods

In order to test *in vitro* the efficacy of anthelmintic substances used for the treatment of hookworms, we conducted a study on 62 dogs living in rural regions of Cluj County, between March and December 2017. We tested *in vitro* the ovicidal and larvicidal effects of four benzimidazole compounds: albendazole (ABZ), mebendazole (MBZ), fenbendazole (FBZ) and *flubendazole* (FLU). We also tested the larvicidal effects of pyrantel (PYR) from tetrahydropyrimidine group as well as selamectin (SEL) from macrocyclic lactone group. The *in vitro* tests were performed by using Egg Hatch Assay (EHA) and Larval Development Assay (LDA). From each individual, fresh faecal samples were collected and a mixed sample was obtained. The Ancylostomatidae eggs were collected from this mixed sample by flotation methods. The techniques were performed within the Pharmacovigilance Laboratory of Cluj-Napoca Veterinary Medicine Faculty as per protocol of Le Jambre (1978).

The total number of eggs per milliliter was determined by using the formula:

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

We used EHA in order to assess the ovicidal properties of the benzimidazole compounds: albendazole (ABZ), mebendazole (MBZ), fenbendazole (FBZ) and *flubendazole* (FLU). For each active drug, serial mother solutions were obtained, ranging from 500 mcg to 0.98 mcg active drug/ml. The samples were placed in 2 ml well plates. For each compound, 10 wells of successively diluted active drugs were used, containing 790 μl of distilled water, 200 μl of egg suspension,

containing about 500 parasite eggs, and 10 μl diluted active drug solutions. The final testing solutions had active drug concentrations ranging from 5.0 to 0.0098 mcg/ml. Two negative controls were used, M_1 consisting of 200 μl egg suspension, containing about 500 parasite eggs, and 800 μl distilled water and M_2 consisting of 790 μl distilled water, 200 μl egg suspension, containing about 500 parasite eggs, and 10 μl of HCl solution. The M_2 control was used only for testing benzimidazoles, because of the fact that these active drugs, have to be dissolved in hydrochloric acid before mixing them with water.

The samples were kept in the thermostat at 27°C for 24 hours and assessed by counting the *embryonated* and *unembryonated* eggs as well as the hatched larvae. The egg hatching percentage was determined applying the formula:

$$\text{Egg hatching \%} = [(EE + L_1) / (UE + EE + L_1)] \times 100$$

(Le Jambre *et al.*, 1978, Coles *et al.*, 1992), where EE signifies the *embryonated* eggs, L_1 represents larvae L_1 and UE signifies *unembryonated* eggs.

Larval development assay (LDA) was used to test larvicidal effects of albendazole (ABZ), mebendazole (MBZ), fenbendazole (FBZ), *flubendazole* (FLU), pyrantel (PYR) and selamectin (SEL). The dilution protocol was identical to the one used for EHA, and the final testing solutions had the same active drug concentrations ranging from 5.0 to 0.0098 mcg/ml. The test had a duration of 14 days in order to culture eggs through the L3 stage. Samples were kept in thermostat at 27°C for 14 days. For this assay the minimum inhibitory concentration (MIC) was obtained by calculating the larval development percentage, applying the formula:

$$\text{Larval development \%} = [(L_3 / (UE + L_3))] \times 100$$

where L_3 represents the number of L_3 larvae and UE stands for *unembryonated* eggs, as per protocol of Coles *et al.* (1988).

The obtained results were statistically interpreted using Anthelmintic Resistance Program (ARP™), a PC software created especially for this type of calculations. ARP calculates and correlates the following parameters: egg hatching percentage,

Table 1. EHA results for the tested benzimidazoles

A.i. conc. (mcg/ml)	ABZ		MBZ		FBZ		FLU	
	Egg hatching %	Regression line equation	Egg hatching %	Regression line equation	Egg hatching %	Regression line equation	Egg hatching %	Regression line equation
5.0000	14.29	66.63	59.52	84.18	46.67	62.62	77.78	88.08
2.5000	50.00	84.38	83.33	92.56	66.67	81.57	77.78	94.28
1.2500	100.00	93.26	96.00	96.74	75.00	91.05	100.00	97.38
0.6250	100.00	97.69	100.00	98.84	100.00	95.78	100.00	98.93
0.3125	100.00	99.91	100.00	99.88	100.00	98.15	100.00	99.71
0.1563	100.00	101.02	100.00	100.41	100.00	99.34	100.00	100.09
0.0781	100.00	101.58	100.00	100.67	100.00	99.93	100.00	100.29
0.0391	100.00	101.85	100.00	100.80	100.00	100.22	100.00	100.38
0.0195	100.00	101.99	100.00	100.86	100.00	100.37	100.00	100.43
0.0098	100.00	102.06	100.00	100.90	100.00	100.45	100.00	100.46
0.0 (M1)	100.00	102.13	100.00	100.93	100.00	100.52	100.00	100.48
0.0 (M2)	100.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
	Parameters		Parameters		Parameters		Parameters	
	a	-7.1	a	-3.35	a	-7.58	a	-2.48
	b	102.13	b	100.93	b	100.52	b	100.48
	Hatching % ref	101.07	Hatching % ref.	100.43	Hatching % ref.	99.38	Hatching % ref.	100.11
	EC ₅₀	7.33450	EC ₅₀	15.18920	EC ₅₀	6.66240	EC ₅₀	20.28040

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

larval development percentage, regression line and its equation (Y parameters), EC₅₀ (the mean concentration that inhibits hatching in 50% of eggs) and MIC (minimum inhibitory concentration). Interpretation of EHA results was obtained by comparing them to those obtained at a reference concentration of 0.15 mcg/ml, as parasite populations in which eggs hatch in a percentage of 50 or above are considered resistant (Cerneza *et al.*, 2010, Coles *et al.*, 1992).

Results and discussions

In EHA, for ABZ high hatching percentages were found in all used concentrations. The minimum value was recorded at 5 mcg/ml concentration, for which the egg hatching percentage was 14.29%. Consequently, the a parameter value was -7.1 and the b parameter 102.13, determining a positive Y max value (66.63) (Table 1).

As a result, the efficacy of ABZ on *Ancylostoma-tidae* eggs is low, this being also highlighted by

the high value of EC₅₀ (7.33450 mcg/ml). The Y parameter values determined for all concentrations used in ABZ, revealed a slightly negative trend of the regression line, which indicates an average risk of installing adaptive phenomena. At the reference concentration of 0.15 mcg/ml, Egg hatching percentage was 101.07%, well above the maximum limit considered as resistance threshold (50%) (Table 1).

A similar situation was also revealed in MBZ testing. The minimum egg hatching percentage was recorded at 5 mcg/ml concentration (59.52%), while at reference concentration, it was 100.43%, showing that MBZ had limited efficacy on the studied helminths. EC₅₀ doubled compared to the one obtained for ABZ (15.189 mcg/ml). The regression line had a slightly negative trend, the value of Y max. parameter being 84.18 (Table 1).

The results obtained with FBZ in the case of EHA have highlighted a situation similar to that found in ABZ and MBZ. The egg hatching percentage

Table 2. LDA results for the tested benzimidazoles

A.i. conc. (mcg/ml)	ABZ		MBZ		FBZ		FLU	
	Larval development %	Regression line equation	Larval development %	Regression line equation	Larval development %	Regression line equation	Larval development %	Regression line equation
5.0000	14.29	33.16	14.29	-64.14	0.00	-27.89	28.00	-1.96
2.5000	0.00	53.41	20.00	-22.32	0.00	-4.42	50.00	28.42
1.2500	50.00	63.54	25.00	-1.40	0.00	7.32	46.67	43.60
0.6250	0.00	68.60	33.33	9.05	10.00	13.19	0.00	51.20
0.3125	42.86	71.13	25.00	14.28	7.14	16.13	27.27	54.99
0.1563	73.33	72.39	20.00	16.90	0.00	17.59	33.33	56.89
0.0781	100.00	73.03	16.67	18.20	10.00	18.33	57.14	57.84
0.0391	71.43	73.34	16.67	18.86	33.33	18.69	66.67	58.31
0.0195	72.73	73.50	16.67	19.18	33.33	18.88	90.00	58.55
0.0098	100.00	73.58	12.50	19.35	14.29	18.97	62.50	58.67
0.0 (M1)	10.00	73.66	10.00	19.51	10.00	19.06	10.00	58.79
0.0 (M2)	60.00	73.66	20.00	19.51	20.00	19.06	60.00	58.79
		MIC (mcg/ml)			MIC (mcg/ml)			MIC (mcg/ml)
		8.46750			0.86720			1.96300
		Parameters			Parameters			Parameters
		a			-8.1			a
		b			73.66			b

Note: A.i. conc. = active drug concentration, MIC = Minimum inhibitory concentration

at reference concentration being 99.38%, with EC₅₀ 6.66240 mcg/ml. The maximum value of Y parameter was 62.62, which implied a slight negative trend for the regression line (Table 1).

The FLU testing for Ancylostomatidae eggs, revealed the worst efficacy compared to the other benzimidazoles. The egg hatching percentage at reference concentration was 100.11%, with EC₅₀ 20.2804 mcg/ml. As a result the regression line tendency ranged between 88.08 and 100 (Table 1).

LDA testing of the benzimidazoles revealed improved efficacy compared to EHA tests. These results are due to the longer contact time between anthelmintic substances and parasitic forms, benzimidazoles having both ovicidal and larvicidal actions (Cernea *et al.*, 2010).

In ABZ testing by LDA, the percentage of larval development inhibition varied according to the active drug concentration, being maximum at 2.5 mcg/ml, respectively 0.625 mcg/ml (0%) concentrations. Minimal percentage of larval development inhibition was recorded at the

concentrations of 0.0781 mcg/ml, respectively 0.0098 mcg/ml (100%). The trend of the regression line, influenced by Y parameter values, was negative, the maximum being 33.16. MIC when tested by using the ARP programme a had a value of 8.4675 mcg/ml (Table 2).

In the case of MBZ testing by LDA, the results obtained are much better compared to those obtained at EHA. It is noted that the prolongation of the contact time between parasitic forms and MBZ determines its high efficacy. The larval development inhibition percentage at the minimal concentration (0.0098 mcg/ml) was 12.50%. The effectiveness of MBZ is also highlighted by the low MIC, which is 0.86720 mcg/ml. Due to the high efficacy of MBZ, the regression line trend is negative, the value of the Y max. parameter being -64.14 (Table 3).

Improvement of anthelmintic efficacy was also noted in the case of FBZ, the MIC being of 1.9630 mcg/ml. Larvae development was completely inhibited at concentrations between 5 and 1.25 mcg/ml, respectively 0.1563 mcg/

Table 3. LDA results for pyrantel and selamectin

A.i. conc. (mcg/ml)	PYR		SEL			
	Larval development %	Regression line equation	Larval development %	Regression line equation		
5.0000	0	-448.37	0.00	-62.74		
2.5000	10	-212.90	0.00	-31.27		
1.2500	10	-95.16	0.00	-15.53		
0.6250	10	-36.29	0.00	-7.66		
0.3125	0	-6.85	0.00	-3.72		
0.1563	0	7.86	12.50	-1.76		
0.0781	20	15.22	25.00	-0.77		
0.0391	0	18.90	40.00	-0.28		
0.0195	20	20.74	66.67	-0.04		
0.0098	60	21.66	50.00	0.09		
0.0000 (M1)	20	22.58	10.00	0.21		
	Parameters		Parameters			
	a	-94.19	MIC - mcg/ml	a	-12.59	MIC - mcg/ml
	b	22.58	0.21310	b	0.21	2.79210

Note: A.i. conc. = active drug concentration, MIC = Minimum inhibitory concentration

ml. Maximum percentages of larval development (33,33%), were recorded at lower concentrations (0.0391 and 0.0195 mcg/ml). The negative value of a parameter (-9,39), influenced the value of Y, that had a maximum of -27.89. Due to these values, the regression line tendency is negative (Table 2).

In the LDA tests, FLU presented the lowest efficacy. The complete inhibition of L3 was noted only at the concentration of 0.6250 mcg/ml. The maximum value of development percentage (90%), was recorded at concentration of 0.0195 mcg/ml. The maximum value of Y parameter was -1.96, and MIC 4.4272 mcg/ml. As a result, although the trend of the regression line was negative, we can assume that in the case of FLU, the parasites from *Ancylostomatidae* family are susceptible to resistance (Table 2).

The best results for the LDA tests, respectively the highest efficacy, were obtained in the case of PYR. The larval development inhibition percentage was maximal at concentrations of 5, 0.3125, 0.1563 and 0.0391 mcg/ml, determining the calculation of a 0.2131 mcg/ml MIC. Compared with other tested active drugs, due to the values of the parameters a and b, Y max parameter had the lowest value (-448.37). Consequently, the regression line had

a strong negative trend, which reveals a low risk of resistance of the studied parasites to PYR-based anthelmintics (Table 3).

SEL completely inhibited the development of L3 larvae at concentrations ranging from 5 to 0.3125 mcg/ml and MIC was 2.7921 mcg/ml. These data show the good efficacy of SEL against parasites from *Ancylostomatidae* family, but lower compared to PYR. The regression line equation recorded for the Y max. parameter a higher value than for PYR (-62.74).

The risk analysis of adaptive phenomena emergence for *Ancylostomatidae* family helminths, determined by the EHA test, reveals that for all tested benzimidazole compounds Y parameter values are with a positive trend between 62.62 and 88.08. As a result, for these active drugs of the benzimidazoles family there is a significant risk of resistance for the isolated parasitic forms.

The results are different in the LDA test, where Y parameter values were negative for MBZ (Y= -64.14), FBZ (Y=-27.89), FLU (Y=-1.96), PYR (Y=-448.37) și SEL (Y=-62.64). Only ABZ has been found to have a positive Y max value (33.16), which impressed a trend towards $+\infty$ for the regression line. Interpretation of these values highlights

the fact that the studied parasitic population is highly susceptible for emergence of ABZ-resistant individuals.

The active drugs with the lowest susceptibility to inducing *resistance* were MBZ, PYR and SEL. Improvement in anthelmintic efficacy has also been noted in FBZ, MIC being 1.9630 mcg/ml and larvae development being completely inhibited at concentrations between 5 and 1.25 mcg/ml, respectively 0.1563 mcg/ml.

The regression lines obtained in EHA and LDA tests, correlate with EC_{50} and MIC values. EC_{50} with the lowest value was obtained for the EHA with FBZ (6.6624 mcg/ml). From this point of view, the lowest efficacy was recorded for FLU (20.2804 mcg/ml) and MBZ (15.1892 mcg/ml).

For PYR test, a concentration of 0.2131 mcg/ml was sufficient to completely inhibit larval development in LDA test. For SEL the MIC was 2.7921 mcg/ml. For the benzimidazole compounds the lowest MIC values were noted at MBZ (0.8672 mcg/ml).

Due to the fact that active drugs with different action mechanisms have different MIC, it is not correct to compare the efficacy of these substances with the resulting MIC. Comparisons can only be made by correlations between EC_{50} , MIC and the regression line trend.

As a result, taking into account the three elements, the highest efficacy and the minimum risk of adaptive phenomena induction for the tested parasites from the Ancylostomatidae family are PYR, SEL, MBZ, and FBZ. ABZ is not recommended to be used for hookworm treatments in the animals used in the study.

The benzimidazoles pharmacological activity is based on the binding to parasite β -tubulin, which produces subsequent disruption of the tubulin-microtubule dynamic equilibrium. Additionally, the inhibition of secretion of nematode acetylcholinesterase and inhibition of some enzymatic activities (such as fumarate reductase, malate dehydrogenase, phosphoenol pyruvate reductase, and succinate dehydrogenase) have been associated with the BZD anthelmintic action (Lacey, 1990; Riviere and Papich, 2018). The fact that the benzimidazoles compounds presented improved efficacy if the parasitic forms are kept longer time in contact with the active ingredients is due to their ovicidal and larvicidal actions, being

stated also by other researches (Cernea *et al.*, 2010, Cristina, 1997).

For albendazole, due to intense liver metabolism and transformation into active metabolites, with the involvement of the cytochrome P450 and flavin monooxygenase systems, efficacy can be much greater when tested *in vivo* compared to *in vitro* (Cernea *et al.*, 2010; Riviere and Papich, 2018).

Selamectin action mechanism against nematodes is by inducing paralytic effects on the parasite somatic musculature and on the pharyngeal pump which affects nutrients ingestion. The paralytic effects are mediated through GABA and glutamate-gated chloride channels. They are considered as extremely effective against adult and larval stages of most gastrointestinal nematodes (Riviere and Papich, 2018).

Selamectin efficacy against hookworms is mentioned in various research in the field of parasitology, being considered as a very good antiparasitic molecule both for dogs and cats (Bishop *et al.*, 2000; McTier *et al.*, 2000; Geurden *et al.*, 2017).

In the case of macrocyclic lactones treatments, the risk of treating MDR1 genetically defective dogs should also be taken into account. Selamectin use is considered safe in ivermectin sensitive Collie dogs (Riviere and Papich, 2018). Changes in the glutamate-gated chloride channels structure and an increased expression of different proteins involved in drug efflux (P-GP) have been postulated as the main mechanism of resistance to macrocyclic lactones in nematodes (Riviere and Papich, 2018). In our research, for the tested parasites, we found no risk in inducing adaptive phenomena against this active drug.

The larvicidal effects of tetrahydropyrimidine compounds are due to their selective action as agonists at synaptic and extrasynaptic nicotinic acetylcholine receptors (nAChR) on nematode muscle cells. They produce contractions and spastic paralysis, mimicking the paralytic effects of excessive acetylcholine amounts. Pyrantel is poorly absorbed from the GI tract. The drug is considered to be highly effective against mature worms and larval stages (Cernea *et al.*, 2015; Riviere and Papich, 2018).

Drug combinations are often recommended for their better efficacy and for lowering the risks of resistance phenomena, often we see

recommendations of macrocyclic lactones, pyrantel and fenbendazole (Wright, 2009; Miro *et al.*, 2007; Clark *et al.*, 1992; Shoop *et al.*, 1996).

On the other hand, empirical use anthelmintics, without consulting the veterinarian, may lead in the near future to the emergence of resistant strains that will complicate the therapeutic protocol, increase the risk of dissemination, and consequently increase the number of infected animals and even people, as these ancylostomatids worms show zoonotic potential (Brenner and Patel, 2003; Croese *et al.*, 1994).

This type of efficacy and resistance prediction tests are recommended to be performed at least once a year due to the ability of parasites to adapt to drug therapy in a relatively short time.

Conclusions

Ovicidal effects of benzimidazole group substances was low, indicating the resistance potential of hookworms to these substances is very high except for FBZ, with lower resistance risk.

The larvicidal effects of benzimidazole group substances, were better compared to the ovicidal effects, with the exception of albendazole.

The risk of inducing resistance phenomena against benzimidazole compounds proved to be lower if the parasitic forms are kept longer time in contact with the active drug testing solutions.

Pyrantel and Selamectin have proven to be very effective against the tested parasites, also presenting minimal risks of resistance phenomena induction. These active drugs, through proven efficacy, are recommended for use as anthelmintics against digestive strongylidoses in the studied dogs, being a good alternative in case of benzimidazole resistance.

Acknowledgments. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

1. Bishop BF, Bruce CI, Evans NA, Goudie AC, Gratton KA, Gibson SP, Pacey MS, Perry DA, Walshe ND, Witty MJ (2000). *Selamectin: a novel broad-spectrum endectocide for dogs and cats*. *Vet Parasitol*, 91:163-176.
2. Brenner MA, Patel MB (2003). Cutaneous larva migrans: the creeping eruption, *Cutis* 72:111-5.
3. Cernea M, Chereji Anca, Catana Laura (2015). *Bazele Farmacologice ale Terapeuticii Medicale Veterinare*, Editura AcademicPres, Cluj-Napoca.
4. Cernea M, Cernea Cristina, Carvalho L (2010). Bio-mathematical software applicable in pharmacological resistance tests, *Scientia parasitologica*, 11(1):24-28.
5. Clark JN, Daurio CP, Plue RE, Wallace DH, Longhofer SL (1992). Efficacy Of Ivermectin And Pyrantel Pamoate Combined In A Chewable Formulation Against Heartworm, Hookworm, And Ascarid Infections In Dogs. *Am J Vet Res*, 53(4):517-20.
6. Coles GC, Tritschler JP, Giordano DJ, Laste NJ, Schmidt AL (1988). Larval development test for detection of anthelmintic resistant nematodes. *Res Vet Sci*, 45: 50-53.
7. Coles GC, Bauer C, Borgsteede FHM, Geerts S, Klei TR, Taylor MA, Waller PJ (1992). World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Vet Parasitol*, 44(1-2):35-44.
8. Cristina RT (1997). *Rezistența trichostrongililor la antihelmintice*. Ph.D. Thesis. Timișoara Faculty of Veterinary Medicine.
9. Croese J, Loukas A, Opdebeeck J et al. (1994). Occult enteric infection by *Ancylostoma caninum*: a previously unrecognized zoonosis. *Gastroenterology*, 106:3-12.
10. Epe C (2009). *Intestinal nematodes: biology and control*. *Vet Clin Small Anim*, 39: 1091-1107.
11. Geurden T, Vatta AF, Nathalie Sloomans, King VL, Lin D, McTier T, Rugg D (2017). Efficacy of a new spot-on formulation of selamectin plus sarolaner against *Ancylostoma tubaeforme* and *Toxocara cati* in cats. *Veterinary Parasitology*, 238: S31-S35.
12. Lacey E (1990). Mode of action of benzimidazoles. *Parasitol today*, 1990(4):112-5.
13. Le Jambre LF, Southcott WH, Dash KM (1978). Effectiveness of broad spectrum anthelmintics against selected lines of *Trichostrongylus columbriformis*. *Aust Vet J*, 54:570-574.
14. Matos Mariana, Ana Margarida Alho, Sinclair PO, Telmo Nunes, LM de Carvalho (2015). Parasite control practices and public perception of parasitic diseases: A survey of dog and cat owners, *Preventive Veterinary Medicine*, 122(1-2):174-180.
15. McTier TL, Shanks DJ, Wren JA, Six RH, Bowman DD, McCall JW, Pengo G, Genchi C, Smothers CD, Rowan TG, Jernigan AD (2000). Efficacy of selamectin against experimentally induced and naturally acquired infections of *Toxocara cati* and *Ancylostoma tubaeforme* in cats. *Veterinary Parasitology*, 91(3-4):311-319.
16. Miro G, Mateo M, Montoya A, Vela E, Calonge R (2007). *Survey Of Intestinal Parasites In Stray Dogs In The Madrid Area And Comparison Of The Efficacy Of Three Anthelmintics In Naturally Infected Dogs*. *Parasitol Res*, 100:317-320.
17. Riviere JE, Papich MG (2018). *Veterinary pharmacology and therapeutics*, Tenth edition, John Wiley & Sons, Inc., Iowa State University Press, USA.
18. Robertson ID, Thompson RC (2002). *Enteric Parasitic Zoonoses Of Domesticated Dogs And Cats*. *Microbes Infect*, 4:867-873.

19. Shoop WI, Michael BF, Soll MD, Clark JN (1996) *Efficacy Of An Ivermectin And Pyrantel Pamoate Combination Against Adult Hookworm, Ancylostoma Braziliense, In Dogs. Aust Vet J.* 73:84-85.
20. Traversa D (2012). Pet roundworms and hookworms: A continuing need for global worming *Parasites & Vectors*, 5:91.
21. Viana Furtado LF, Ana Cristina Passos de Paiva Bello, Hudson Andradedos Santos, Maria Raquel Santos Carvalho, Élide Mara Leite Rabelo (2014). First identification of the F200Y SNP in the β -tubulin gene linked to benzimidazole resistance in *Ancylostoma caninum*. *Veterinary Parasitology*, 206(3-4): 313-331.
22. World Health Organization (2011). *World Malaria Report 2011*. Who; Geneva, Switzerland: 2011
23. Wright I (2009). Canine hookworm: a worm for all climates, *UK Vet*, 14(7):1-4.