

**THESIS OF DOCTORAL (PhD)
DISSERTATION**

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**BENZIMIDAZOLE RESISTANCE IN *HAEMONCHUS CONTORTUS* IN
DOMESTICATED AND WILD RUMINANT POPULATIONS IN SOUTHWESTERN
HUNGARY**

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ANTECEDENTS AND OBJECTIVES OF THE DISSERTATION

Arguably, the presence of *Haemonchus contortus* (barber's pole worm) populations is one of the most important threatening factors in the small ruminant sector. Due to its high pathogenicity and the unique phenotypic plasticity driven by a considerable genetic diversity, this parasite causes huge production losses and socio-economic problems globally, each year. Although this bloodsucking helminth is originated as a tropical species, today, however, its spatial distribution covers all of the climatic zones excepting the polar regions. The climate change, raising mean temperature and other weather anomalies can provide more and more capable habitats for surviving of free-living stages.

Decrease the losses caused by GIN, anthelmintic treatments and chemo-prophylaxis could be efficacious tools. Until now, just a few broad-spectrum drug classes are available for control. The oldest one, benzimidazoles, was introduced almost 60 years ago, while the youngest one, amino-acetonitrile derivative, is accessible since 2008. Unfortunately, these relatively inexpensive drugs became the only weapon in the battle against the endoparasites. Their solely and frequent application resulted in the occurrence and spread of AR within a few years in every class. Nevertheless, these obvious facts are well-known in several regions of the World; the level of AR in Hungarian sheep sector is absolutely unidentified. The lack of this information can cause not just economic and animal health problem in the sector but hampers the efficient GIN control.

The molecular diagnostic methods are very useful tools for a rapid and accurate approach of the routine diagnosis of GIN infections in the sheep farming practice. They could combine with conventional tests (e.g. FECRT, in vitro assays) to recognise the early appearance and dispersion of AR in the worm populations.

The grazing lands are fundamental resources of the small ruminant production systems. Due to their naturalness, the included habitats are shared many times between wild and domestic ungulate species. This mutual exploitation can generate the potential for transmission of parasitic nematodes between host populations. Studies of GIN transmission at the wildlife/livestock interface rarely discussed; although they could induce strong effects in health, production, and population dynamics. Some studies verified the possibility of *H. contortus* cross-infection between different types of ruminants in natural circumstances. Confirmed by genotyping (Cerutti et al., 2010), *H. contortus* strains were well mixed between wild and domestic ungulate hosts, and it was concluded this transmission occurs in shared pastures regularly.

To date, few studies have been accomplished to confirm the potential role of cervids in carrying and spreading of AR nematodes. AR *H. contortus* were found in free-living, untreated roe deer and successfully transmitted experimentally to sheep (*Ovis aries*) and cattle (*Bos taurus*). These results indicating, that deer could contribute to the spread of AR in natural circumstances.

Hungarian sheep farming has a very important socio-economic role, mainly in the less-developed rural region. For sufficient incomes, the farmers should maintain well-managed flocks. In this process, the parasite control has a core role. Unfortunately, parasitological status and the level of AR in Hungarian sheep sector are absolutely unidentified. The lack of this information can cause not only economic and animal health problem in the sector but hamstrings the efficient GIN control.

The Game Management Landscape Centre of the Kaposvár University has one of the most important red deer (*Cervus elaphus*) stocks in Middle-Europe. In 1300 hectare, more than 1500 red deer are managed for venison and hunting purposes. This animal density is much higher than in the neighbouring natural habitats. Without drug application against micro and macroparasites, the economic aims proposed by the management are unimaginable.

Our co-operation with the SEFAG Forest Management and Wood Industry Share Co. dates back to 2007. In its framework, we have been monitoring the stock of game managed by the firm. This surveillance covers all of the hunting areas and their wild boar (*Sus scrofa*), red deer, fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), mouflon (*Ovis aries orientalis*), red fox (*Vulpes vulpes*), and golden jackal (*Canis aureus*) populations. Due to this activity, we picked up information about several pathogens are existing in the concerned areas. As the headquarter has a One Health approach in wildlife management, a study was planned to clarify whether parasitic diseases are existing in the wild populations and could infect the domesticated animals and humans. (*One World – One Health – One Medicine* (briefly *One Health*) approach that amalgamates skills of both epidemiology and ecology serves additional information. Application of ecological methods during investigation on a parasitic or an infectious disease is more useful than simple diagnostic investigation of reservoir species and statistical evaluation of prevalence and intensity data.) In the frame of this cooperation, the *Haemonchus contortus* populations were studied in red and roe deer.

Our principal objective was to determine the frequency of benzimidazole resistance in different ruminant species. For this reason, a parasitological and a molecular diagnostic method (RFLP-PCR) were conducted in *Haemonchus contortus* specimens derived from sheep, farmed and free-ranging red deer and roe deer flocks.

An additional objective was to evaluate the role of free-ranging deer species in the spread of anthelmintic resistance.

METHODOLOGICAL SUMMARY

In our work, we studied the level of resistance in barber's pole worm isolated in different host populations and assessed the possible role of wild ruminants in the spread of resistance. For this reason, we collected and dissected sheep, red deer, and roe deer abomasa in the southwestern part of Hungary. For implementing the aims, four studies were conducted and focused the analysis of codon 200 of β -tubulin gene isotype 1.

In Study 1 and Study 2, domesticated ruminant flocks were surveyed. In the third investigation, free-ranging cervids, and sheep flocks were compared to determine the AR level in their *H. contortus* populations. In the last study, the possible role of roe deer was assessed in the spread of AR. The connections and relevant information between the research elements are shown in Figure 1.

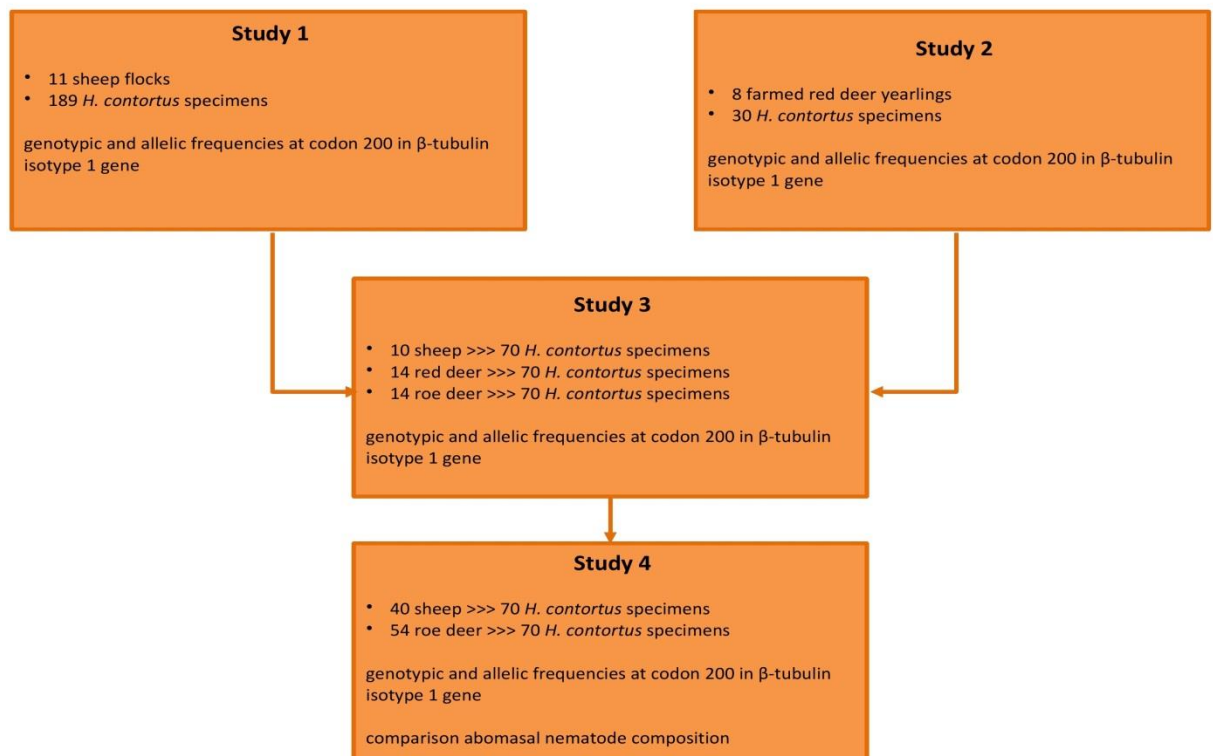


Figure 1. Flowchart of the studies

RESULTS

Study 1

A total of 189 male *H. contortus* were examined from 11 different, southern Transdanubian sheep flocks. The BZ resistance was detected at codon 200 in β -tubulin isotype 1 gene. The occurrence of the three genotypes and the allele frequency showed a wide variety among the flocks (Table 1). The summarised allele frequencies were 36.24% (susceptible: S) and 63.76% (resistant: R), respectively. The occurrence of homozygous susceptible (SS) and heterozygous (RS) was similar (23.28% and 25.93%, respectively), and the proportion of homozygous resistant (RR) was about twice as much (50.79%).

Table 1. Occurrence of different genotypic and the frequency of resistant and susceptible alleles in flocks

flock	sample size	genotypic frequency (%)			allele frequency (%)	
		SS*	RS	RR	S	R
No.1	15	0	30	70	16.7	83.3
No.2	15	0	26.7	73.3	13.3	86.7
No.3	15	0	20	80	10	90
No.4	15	0	26.7	73.3	13.3	86.7
No.5	17	11.8	58.8	29.4	41.2	58.8
No.6	18	0	27.8	72.2	13.9	86.1
No.7	20	100	0	0	100	0
No.8	17	11.8	58.8	29.4	41.2	58.8
No.9	20	15	40	45	35	65
No.10	17	100	0	0	100	0
No.11	20	0	0	100	0	100
Sum	189	23.28	25.93	50.79	36.24	63.76

* SS: homozygous susceptible; RS: heterozygous; RR: homozygous resistant

We assessed the linear correlation between BZ usage and R allele frequency by a questionnaire was filled by farmers or their veterinaries. Information was collected about the average annual frequency of treatments in the past 3 years and since when the farmers have been using BZ. Strong correlation coefficients were found between the variables annual and both connections proved to pronouncedly significant.

Study 2

Analysis of 30 *H. contortus* specimens was carried out to detect BZ resistance at codon 200. Among the worms, 21 were genotyped as homozygous resistant (RR) and nine as heterozygous (RS). True prevalence of RR genotype was proved to be 46.5–87.2% (confidence interval: 95%) calculated by Sterne's exact method. Calculated by this method,

SS genotype and S allele showed 0.0–11.2% and 7.8–26.5% true prevalence, respectively. We found that proportion of RR worms and the frequency of resistant (R) allele were very high in the worm population (Table 2).

Table 2. Genotypic allele frequency in male *Haemonchus contortus* (S=susceptible, R=resistant) in Study 2; N=30

Genotypic frequency (%)		Allele frequency (%)		
homozygous resistant (RR)	heterozygous (RS)	homozygous susceptible (SS)	resistant (R)	susceptible (S)
70	30	0	85	15

Study 3

In this study, we examined altogether 210 male worms, which were identified as *Haemonchus contortus*, to determine the proportion of Phe200Tyr SNP on codon 200 of β -tubulin gene isotype 1 within three different ruminant species. Among the worms, 110 were genotyped as homozygous susceptible (SS), 40 as heterozygous (RS) and 60 as homozygous resistant (RR), thus the total frequency of SS, RS and RR was 52.4% (CI95% = 45.5-59.1%), 19% (CI95% = 14.1-24.9%) and 28.6% (CI95% = 22.7-35.2%), respectively. Considering the alleles, the proportion of susceptible (S) and resistant (R) allele was 61.9% (CI95% = 57.2-66.5%) and 38.1% (CI95% = 33.5-42.8%), respectively. Distribution of the different genotype was showed a wide variety in hosts. The SS was the most representative in the red deer, where all of the worms belonged to this genotype group. The RR was most prevalent in the sheep and it was moderate in the roe deer, while the RS was observed in equal proportion in these two host (Table 3). Difference of allele frequencies between the host populations was confirmed significant by Chi-squared test.

Table 3. Genotype and allele frequencies (CI95%) in *Haemonchus contortus* (S=susceptible, R=resistant) collected during Study 3.

Host	Genotype frequency (%)		Allele frequency (%)		
	homozygous susceptible (SS)	heterozygous (RS)	homozygous resistant (RR)	susceptible (S)	resistant (R)
red deer (N=70)	100 (94.9-100)	0 (0-5.1)	0 (0-5.1)	100 (97.4-100)	0 (0-2.6)
roe deer (N=70)	54.3 (42.4-65.9)	28.6 (18.9-40.6)	17.1 (9.7-27.6)	68.6 (60.4-75.9)	31.4 (24.18-39.6)
sheep (N=70)	2.90 (0.5-9.5)	28.6 (18.9-40.6)	68.6 (56.5-78.9)	17.1 (11.6-24.1)	82.9 (75.9-88.4)

Study 4

The roe deer had more diverse abomasal fauna, than the sheep had. Seven of the worms were proved to be a dominant or codominant species; the rests belonged to subordinate ones. We found just two common worm species but just *H. contortus* was considered as a dominant in both ruminant populations (Table 4)

Table 4. Importance values of nematode species by hosts

Nematode	Roe deer	Sheep
<i>Ashworthius sidemi</i>	0.13 ^{CD}	0
<i>Haemonchus contortus</i>	21.67 ^D	43.99 ^D
<i>Teladorsagia circumcincta/T. trifurcata</i>	0.001 ^{>}	43.94 ^D
<i>Spiculoptera spiculoptera/S. mathevossiani</i>	17.21 ^D	0
<i>Spiculoptera asymmetrica/S. quadrispiculata</i>	0.17 ^{CD}	0
<i>Ostertagia leptospicularis/O. kolchida</i>	34.3 ^D	0
<i>Ostertagia ostertagi/O. lyrata</i>	0.009	0
<i>Trichostrongylus axei</i>	0.004	0
<i>Nematodirus oiratianus</i> subsp. <i>interruptus</i>	0.004	0

(D) indicate dominant species, while (CD) does codominant ones

We performed a genetic analysis using 140 male *H. contortus* male specimens. The homozygous susceptible (SS) genotype was the more representative in the roe deer (54.3%), than in sheep (2.9%). On the other hand, the homozygous resistant (RR) worms were most prevalent in the sheep (68.6%) and were moderate in the roe deer (17.1%), while the heterozygous (RS) genotype was observed in equal proportion in both hosts (28.6%). Difference of allele frequencies (roe deer: susceptible allele = 68.6%; resistant allele = 31.4%; sheep: susceptible allele = 17.1%; resistant allele = 82.9%) between the host populations was confirmed as significant ($p < 0.05$).

CONCLUSIONS

The Study 1 revealed that the presence of homozygous BZ resistant *H. contours* is high (50.79%) The frequency showed a strong correlation with the treatment frequency ($R=0.7674$; $p=0.0058$) and the long-term use ($R=0.7789$; $p=0.0047$) of BZ.

In Study 2, a similarly prominent resistance level (70%) was also observed in a farmed red deer population. It is the first genetic evidence of BZ resistance in *H. contortus* in the red deer. There has not been any rotation of unrelated anthelmintics in this herd to delay AR. It is likely that routine use of ABZ for many years has favoured the survival of the resistant genotype, thus inducing spread of R allele in this *H. contortus* population. In this case, the speed of applied ABZ metabolism could contribute to the high resistance level. The characteristic of microsomal liver metabolism in deer strongly suggests that these species require a higher dose rate than sheep and cattle to attain optimal efficacy rates against susceptible parasites. This organ, among other factors, affects the rate of metabolism and the active level of drug within the host organism. Owing to the liver microsomal enzymes (e.g. cytochrome P450), the red deer detoxicates its organism much more effectively than sheep. Therefore the correct sheep or cattle dosing is inadequate to eliminate the nematodes from deer. It causes a suboptimal-dosage and accelerates the dominance of resistant allele, and contributes to the selection of a resistant worm population.

The molecular test of Study 3 confirmed a moderate frequency (31.4%) and absence of R allele in roe deer and red deer, respectively. By these results, it seems that in the spread of BZ resistance, the roe deer could play a role. This theory is proved by the parasitological examination of Study 4. In this case, only the *H. contortus* was the dominant species in both hosts. Notwithstanding, its importance was not so remarkable in the cervids (21.67) as in the sheep (43.99). These findings suggest that rather the sheep farming affects the roe deer

habitat, than vice versa. The comparison of AR prevalence in the two hosts also supports the superiority of human influence. Within the studied habitat, antiparasitic treatment of wildlife has never been in practice. In spite of this, AR is present in the parasite community of the roe deer; though its level is much lower than in the sheep. These results support the hypothesis, that habitat overlapping between sheep and roe deer in this area means rather an advantage than a real risk for antiparasitic strategies. Worm community excreted by wild ruminants contains fewer AR individuals and more competitively superior, non-blood-sucking species. The helminths they shed behave like a buffer in the refugia and may dilute the R allele frequency in the shared pastures.

NEW SCIENTIFIC RESULTS

1. We have found high benzimidazole resistant allele frequency (63.76%) in *Haemonchus contortus* in sheep flocks. This incidence shows a strong linear correlation with treatment frequency and length of anthelmintic usage.
2. We have found high benzimidazole resistant allele frequency (85%) in *Haemonchus contortus* in a farmed red deer flock.
3. We have found a high (82.9%) a moderate (31.4%) and a lack of benzimidazole resistant allele frequency in *Haemonchus contortus* in sheep flocks and free-living roe and red deer populations, respectively, in a natural habitat shared by them.
4. *Haemonchus contortus* was considered as a dominant nematode species in free-living roe deer and the sympatric sheep populations.
5. We have confirmed the presence of *Ashworthius sidemi* and *Nematodirus oiratianus* subsp. *interruptus* in Hungary.

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