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Situation of benzimidazole resistance in *Haemonchus contortus* in southwestern Hungary

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ABSTRACT

Among the gastrointestinal nematodes of small ruminants Haemonchus contortus *has almost the most overwhelming importance. This abomasal bloodsucking parasite has been presented all over the world, and it causes enormous economic and health problems in the sheep sector. A total of 189 adult male* H. contortus *worms were collected from sheep, bred southwestern Transdanubian region of Hungary, for monitoring whether the long-term usage of benzimidazoles could affect their effectiveness. The summarised allele frequencies, analyzed by RFLP-PCR, were 36.24% and 63.76% in case of susceptible and resistant ones, respectively. The proportion of homozygous susceptible (23.28%) and heterozygous (25.93%) worms were similar and the portion of homozygous resistant was about twice as much (50.79%). The correlation was pronouncedly significant between resistance allele frequency and the usage of benzimidazoles. According to our results, it seems the BZ resistance has appeared and extended within* Haemonchus contortus *in Hungarian sheep flocks.*

(Keywords: *Haemonchus contortus*, Hungary, sheep, benzimidazole resistance)

INTRODUCTION

Among the gastrointestinal nematodes of small ruminants *Haemonchus contortus* has almost the most overwhelming importance. This abomasal bloodsucking parasite has been presented all over the world, including in Hungary; and it causes enormous economic and health problems in the sheep sector (*Waller and Chandrawathani*, 2005).

The treatment of gastrointestinal nematode infections could be feasible by broad-spectrum anthelmintics, which can be divided into three groups such as benzimidazoles (BZ), macrocyclic lactones (ML), imidazothiazoles (IT). In the recent years, it has seemed that the effectiveness of these drugs, mainly BZ and ML, reduced in many Hungarian sheep and goat flocks.

BZs are the most majorly used anthelmintics, due to their advantageous properties; such as high therapeutic index, the absence of toxic residuals in milk and meat and economical availability (*Tiwari et al*., 2006). BZ resistance in *H. contortus* is associated with single-nucleotide polymorphisms (SNP) on codon 167, 198, and 200 of β-tubulin isotype 1 gene (*Mottier and Prichard*, 2008). The most relevant diagnostic tool is the detection of Phe200Tyr SNP on codon 200 (*Coles et al.,* 2006). The main advantages of molecular diagnostics are sensitivity and accuracy; therefore, even a low frequency of resistant alleles can be detected. On the other hand, comprehensive application of these methods is impeded by expenses.

Many factors could facilitate the occurrence and spread of anthelmintic resistance (AR) in worm populations. However; probably the most important one is the inadequate usage of drugs. The frequent usage of anthelmintics may result the development of AR (*Waller*, 1997).

Rigid defensive strategies, wherein the number of treatments may consist of 5 or more occasions, and a very strong selective pressure could modify the worm populations. The frequent drug usage supports the surviving of such parasites; which possesses resistance allele. By the continuous selection in the nematode population, the occurrence of resistance allele (R) could be dominant; and the susceptible allele (S) could be restricted, thus the given anthelmintics may lose their effectiveness.

The sub-optimal dosage may also play a role in AR development (*Smith*, 1990). The underdosing promotes the survival of not just homozygous resistant (RR) but heterozygous (RS) parasites. These fault treatments eliminate just homozygous susceptible (SS) specimens and result a domination of the R allele.

The long-term use of anthelmintics could contribute the increasing of AR level. The continuous usage of a given drug creates a selection against S, as it was shown in the case of frequent treatments. Some authors interpreted that permanent usage of BZ and ML without rotation has resulted AR in *H. contorus* in South Africa (*van Wyk et al*., 1988).

In Hungarian veterinary practice, one of the most preferred drug groups is BZ, which has been presenting in the market for several decades. The aim of our study was, whether the long-term usage of these anthelmintics could affect their effectiveness of worm control in the Hungarian small ruminant sector.

MATERIAL AND METHODS

Collection of parasites

Adult male *H. contortus* worms were collected from sheep flocks in the southwestern Transdanubian region of Hungary (*Figure 1*).

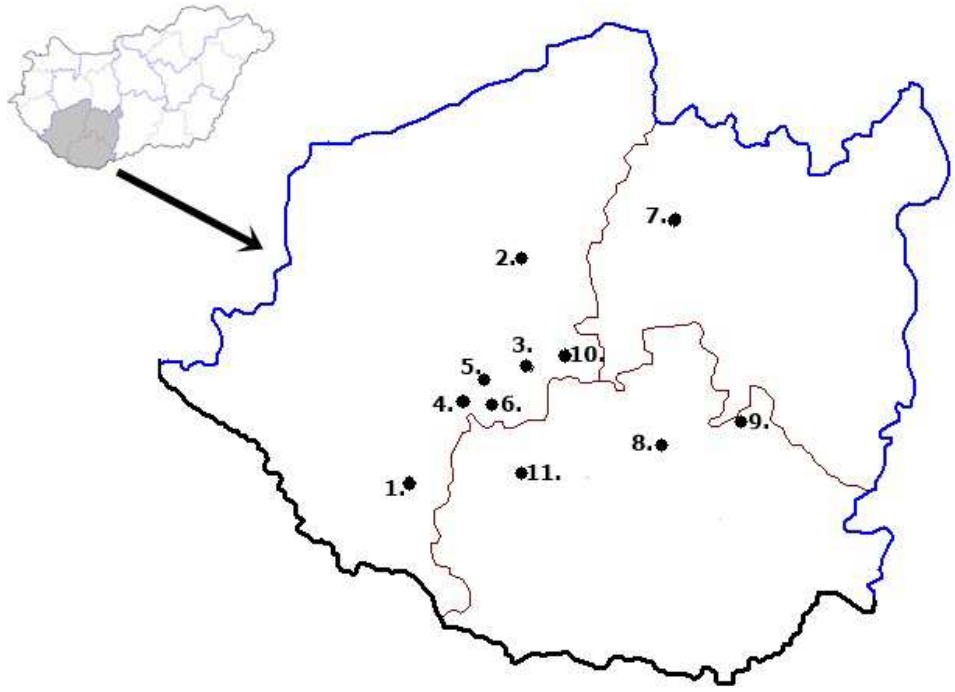

Figure 1.

Localization of sheep flocks

The 189 specimens were isolated either from a regional abattoir, perished animals from the farms or during diagnostical necropsies from the diagnostic veterinary institute. In every case, an abomasal dissection was carried out as soon as possible after death, when we cut the organ

alongside the big curvature and placed in a plastic bucket filled saline solution. The worm collection was performed in a veterinary laboratory, where we washed thoroughly the abomasum mucosa and left the content to consolidate. After 5 minutes, the supernatant was decantated. This process was repeated till the supernatant had become lucid.

The worms were collected by their "barber pole" characteristics. The species identification was performed by a light microscope with 100X magnification, using the work of *Lichtenfels et al*. (1994). Until genotyping, the isolated parasites were kept in 96% alcohol.

DNA extraction and genotyping

The genotypic analysis was carried out by Restriction Fragment Length Polymorphism-Polymerase Chain Reaction (PCR). The applied primer sequences were as follows: AvikaF : 5'- CTA CCCTTTCCGTCCATCAA -3' and AvikaR: 5'- TGAAGACGAGGGAATGGAAC -3' (*Tiwari et al*., 2006). Primers were designed to amplify a 303 bp fragment using DNA sequence of β-tubulin isotype 1 gene. PCR reactions were performed in a total volume of 10 µl, containing 200 µM of each dNTP, 0.2 µM primers, 10 × PCR buffer, 0.5 unit Dynazyme DNA polymerase and 100 ng genomic DNA. The PCR cycling profile consisted of denaturation at 94°C for 3 min, 45 cycles of denaturation at 94°C (for 30 sec), annealing at 56 °C (for 30 min), and extension at 72°C (for 30 min), followed by a final extension at 72°C for 5 minutes. Digested fragments (by TaaI endonuclease) were resolved in 4% agarose gel stained with SYBR® Green II Nucleic Acid Gel Stain and visualised under UV light. Genotypes were determined based on the fragment lengths such as 305 bp S allele and 257 bp for R allele.

Data collection and statistical analysis

The genotypic and allelic frequencies were determined by GenAlEx software 6.502 version (*Peakall and Smouse*, 2012) separately in every flock and all together.

In order to determine the linear correlation between BZ usage and R allele frequency (RALL), we have had a questionnaire was filled by farmers or their veterinaries. Information was collected about the average annual frequency of treatments in the past 3 years (BZAT) and since when the farmers have been using BZ (SBZU). The correlation was determined between variables by R statistical software, version 3.3.0 (https://www.r-project.org/).

RESULTS AND DISCUSSION

We examined a total of 189 male *H. contortus* derived 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% (S) and 63.76% (R), respectively. The occurrence of SS and RS was similar (23.28% and 25.93%, respectively), and the proportion of RR was about twice as much (50.79%).

The homozygous susceptible genotype was observed in 5 flocks (2 flocks were SS in 100%). We found just a flock where all the collected worms were homozygous resistant. The correlation coefficients were very similar between the variables (RALL and BZAT: 0.7674; RALL and SBZU: 0.7789) and both connections proved to pronouncedly significant also (RALL and BZAT: p=0.0058; RALL and SBZU: 0.0047).

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

Flock	Sample size	Genotipic 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	11.76	58.83	29.41	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

The emerging of AR in several nematodes of ruminant species is known all over the world, including Europe (*Kaplan*, 2004; *Ihler*, 2010; *Papadopoulos et al*., 2012). However; till now there was not any information on BZ resistance in Hungary, though our study showed its presence. One of the most influential factors in the occurrence of AR is the usage method of anthelmintics. It is well known that the intensive chemical treatments exclusively could not assist a long-term protection against worms. The continuous drug application, without any rotation, could facilitate the increasing of resistance level in helminth populations (*Waller*, 1997; *Jabbar et al*., 2006). The results of our genetic and statistical analysis confirmed a strong linear correlation between R allele frequency and the treatment frequency and the length of BZ usage. In a study, *Calvete et al*. (2010) analysed the management and environmental factors related to benzimidazole resistance, in Northeast Spain. Applying a principal component analysis, the authors suggested, that frequency of deworming was the single management variable that increased the BZ resistance level in the worm populations.

By our result, we suggest the farmers, practitioners, experts, and veterinaries to change their approach in connection with anthelmintic strategies. They should form novel, integrated, complex and sustainable methods, which contain more actions to fight against worms, for instance resistance breeding, environmental and immunological control, improved pasture and nutritional management, target selective treatment and the refugia management (*van Wyk*, 2001; *van Wyk et al*., 2006, *Kenyon et al*., 2009, *Bath*, 2014).

CONCLUSION

According to our results, it seems the BZ resistance has appeared and extended within *Haemonchus contortus* in Hungarian sheep flocks. We hypothesise, that long-term usage and the recurrent anthelmintic treatments could be in the background of pronounced proportion of resistant allele. Therefore; we strongly recommend the farmers, practitioners, experts, and veterinaries, to change their own approaches to chemical protection. They need to apply a more complex and integrated defending strategy against gastrointestinal nematodes in order to prepare an effective parasite control management.

REFERENCES

Bath, G.F. (2014) The „Big Five" – A South African perspective on sustainable holistic internal parasite management in sheep and goats. Small Ruminant Res 118 (1-3). 48-55.

Calvete, C., Calavia, R., Frrer, L.M., Ramos, J.J., Lacasta, D., Uriarte, J. (2010) Management and environmental factors related to benzimidazole resistance in sheep nematodes in Northeast Spain. Vet Parasitol 184. 193-203.

Coles, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M.A., Vercruysse, J. (2006) The detection of anthelmintic resistance in nematodes of veterinary importance. Vet Parasitol 136 (3-4). 167–185.

Ihler, C.F. (2010) Anthelmintic resistance. An overwiev of the situation in the Nordic countries. Acta Vet Scand 52 (1). 1-5.

Jabbar, A., Iqbal, Z., Kerboeuf, D., Muhammad, G., Khan, M.N., Afaq, M. (2006) Anthelmintic resistance: The state of play revisited. Vet Parasitol 79. 2413-2431.

Kaplan, R.M. (2004) Drug resistance in nematodes of veterinary importance: astatus report. Trends Parasitol 20 (10). 477-481.

Kenyon, F., Greer, A.W., Coles, G.C., Crignoli, G., Papadopoulos, E., Cabaret, J., Berrag, B., Varady, M., van Wyk, J.A., Thomas, E., Vercruysse, J., Jackson, F. (2009) The role of targeted selective treatments in the development of refugia-based approaches to the control of gastrointestinal nematodes of small ruminants. Vet Parasitol 164 (1). 3-11.

Lichtenfels, J.R. Pilitt, P,A. Hoberg, E.P. (1994) New morphological characters for identifying individual specimens of Haemonchus spp. (Nematoda: Trichostrongyloidea) and a key to species in ruminants in North America. J Parasitol 80 (1). 107-119.

Mottier, M.L., Prichard, R.K. (2008) Genetic analysis of a relationship between macrocyclic lactone and benzimidazole anthelmintic selection on *Haemonchus contortus*. Pharmacogenet Genom 18. 129-140.

Papadopoulos, E., Gallidis, E., Ptochos, S. (2012) Anthelmintic resistance in sheep in Europe: A selected review. Vet Parasitol 189. 85-88.

Peakall, R. and Smouse, P.E. (2012) GenAlEx 6.5: genetic analysis in Excel. population genetic software for teaching and research – an update. Bioinformatics 28. 2537-2539.

Smith, G. (1990) Chemotherapy:future problems. In: Schad, G.A., Warren, K.S. (Editors), Hookworm disease: Current status and new directions. Taylor & Francis, London, UK, 291-303.

Tiwari, J. Kumar, S. Kolte, A.P. Swarnkar, C.P. Singh, D. Pathak, K.M.L. (2006) Detection of benzimidazole resistance in *Haemonchus contortus* using RFLP-PCR technique. Vet Parasitol 138 (3-4). 301–307.

van Wyk, J.A. (2001) Rfeugia-overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. Onderstepoort J Vet Res 68 (1). 55-67.

van Wyk, J.A. Malan, F.S. (1988) Resistance of field strains of *Haemonchus contorus* to ivermectin, closantel, rafoxanide and the benzimidazoles in South Africa. Vet Record 123 (9). 226-228.

van Wyk, J.A., Hoste, H., Kaplan, R.M., Besier, R.B. (2006) Target selective treatment for worm management – How do we shall rational programs for farmers. 139 (4). 336-346.

Waller, P.J. (1997) Anthelmintic resistance. Vet Parasitol 72 (3-4). 391-412.

Waller, P.J. and Chandrawathani, P. (2005) *Haemonchus contortus*: Parasite problem No.1 from tropics-polar circle. Problems and prospects for control based on epidemiology. Trop Biomed 22 (2). 131-137.

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