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Benzimidazole resistance within red deer, roe deer and sheep populations within a joint habitat in Hungary

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ABSTRACT

The anthelmintic resistance of gastrointestinal nematodes is one of the most important, economic risk factors in grazing ruminant systems, all over the world. We have infinitesimal information about the resistance status of nematodes in deer species. Our aim was to determine the presence of BZ resistance in the generalist worm, *Haemonchus contortus* in pastured sheep and free ranging red and roe deer by RFLP-PCR method based on the detection Phe200Tyr single nucleotide polymorphism. By investigation of 70 worms from each host species, the homozygous susceptible genotype was the most representative in the red deer (100%), the homozygous resistant genotype was most prevalent in the sheep (68.6%) and moderate in the roe deer (17.1%), while the heterozygous genotype was observed in equal proportion in the sheep and roe deer (28.6%). Our results suggest that overlapping habitats of sheep flocks and roe deer could contribute to the occurrence and spread of resistant allele within wildlife.

1. Introduction

The anthelmintic resistance (AR) of gastrointestinal nematodes is one of the most important economic risk factors in grazing ruminant systems all over the world (Sutherland and Leathwick, 2011; Papadopoulos et al., 2012; Rose et al., 2015). The first appearing of resistance against broad spectrum drugs dates back to the 1960's and its spreading could be observed in every class (FAO, 2004; Kaplan, 2004).

Although, the phenomenon of AR is well known in major domesticated ruminant species, as in the case of sheep (Barrere et al., 2013; Peña-Espinoza et al., 2014; Chaudhry et al., 2015); within the minor food-producing species (eg. deer species), we have much less information about the AR status of their nematodes. In a study (Mackintosh et al., 2014), the resistance to moxidectin and abamectin was demonstrated by faecal egg count reduction test (FECRT) in red deer. The authors suggested that AR of *Ostertagia*-type species was in the background of drug ineffectiveness. A similar result was showed in the case of albendazole and ivermectin (Mylrea et al., 1991) in fallow deer. Both studies were carried out under farm circumstances. It was hypothesized that failure of treatments was affected by several factors including the AR of gastrointestinal nematodes. Chintoan-Uta et al. (2014) tested and confirmed the cross transmission of *Haemonchus contortus* and its benzimidazole (BZ) resistance between the fallow deer, red deer, roe deer, cattle, and sheep. Both of their resistance detection methods (egg hatch test and molecular test) demonstrated the presence of AR in the worms derived from roe deer. Three single nucleotide polymorphisms (SNPs) in the β-tubulin gene isotype 1 have been associated with benzimidazole resistance in *H. contortus*. The Phe200Tyr mutation has been found in every country at a relatively high frequency. The SNPs at codons 167 and 198 have also been reported in multiple countries but have a more variable occurrence and are generally present at a lower frequency than the Phe200Tyr mutation (Chaudhry et al., 2016). Since the most common molecular mechanism that confers BZ resistance in trichostrongyles in small ruminants involves a phenylalanine to tyrosine mutation at codon 200 of β-tubulin gene isotype 1; therefore, molecular diagnostic methods based on the detection of this SNP are used most frequently to identify BZ resistance in *H. contortus* (Coles et al., 2006).

A Hungarian study (Nagy et al., 2016) demonstrated a high homozygous resistant genotype (70%) and resistance allele (85%) proportion in farmed red deer. The results of a molecular diagnostic method showed that routine, long-term administration of the

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same drug could contribute to the appearance of AR in *H. contortus* in the red deer.

Recently, a new approach, refugia theory, arose during the investigation of AR in natural environment. It hypothesizes that the presence of wild ruminants on pastures of livestock provides a significant percentage of homozygous sensitive ones among the total amount of infectious larvae. Regular infection by this mixed population impedes the unlimited spread of resistance within a worm population infecting a livestock flock concerned. (Van Wyk, 2001).

In this study, we aimed to determine the presence of BZ resistance in the generalist worm, *Haemonchus contortus* in a microregion, where the resources partly shared by sheep and free ranging red and roe deer.

2. Materials and methods

2.1. Study site

Our investigation was carried out in southwestern Hungary, within a contiguous area without any natural or man-built isolating elements that impede movements of animals (Fig. 1). The study site (approximately 30 km^2) characterized by 145–276 m altitude above sea level, sub-Mediterranean climate, with some submontaneous habitat patches, with 10 °C annual mean temperature and 630–800 mm annual precipitation. The proportion of forests and agricultural areas on study site are about 55% and 45%, respectively. In the core of the site, a 6000-ha monoblock forest exists with the domination of oaks (*Quercus* spp.), limes (*Tilia* spp.), hornbeam (*Carpinus betulus*) and European beech (*Fagus sylvatica*). The agricultural areas (viz. grasslands, pastures, arable lands, and old orchards) are the most typical on the periphery of the study site and they provide a heavily fragmented landscape structure. The average density of red deer, roe deer, and sheep are 1.71, 0.84 and 0.17 animal/km^2, respectively. Our data were based on hunting statistics (hunted deer specimen/km^2); while in the case of sheep, it was calculated by the official registry of the Hungarian Sheep and Goat Breeders Association.

2.2. Worm collection and molecular diagnostic procedure

During the interval from September of 2013 till August of 2015, we collected 38 abomasi from red deer (N = 14), roe deer (N = 14) and sheep (N = 10 from 5 different flocks). The organs of deer were collected from hunting bags in regular hunting seasons, while sheep were sampled at a regional abattoir. After evisceration, each abomasum was placed separately into a plastic bag immediately, and each was stored at −18 °C until examination. After melting, the organs were opened alongside the big curvature, and the content was placed into a plastic jar, while the mucosa was washed thoroughly. In the case of deer species, 5 *Haemonchus contortus* males were picked randomly from each organ, while 7 specimens were collected from every single sheep abomasum. For morphological identification, we used Lichtenfels's et al. (1994) work. That method is based on the length of the left and right spicule barbs.

In order to genotype the collected worms, we used the detection of Phe200Tyr single-nucleotide polymorphisms on codon 200 of β-tubulin gene isotype 1, which is the most common molecular mechanism conferring BZ resistance in trichostrongyles of small ruminants (Coles et al., 2006). DNA lysates were made separately from 210 adult male worms. The applied Restriction Fragment Length Polymorphism-PCR method and the primer sequences (AvikaF: 5′-CTA CCCTTTCCGTCCATCAA -3′ and AvikaR: 5′- TGAAGACGAGGGAATGGAAC -3′) were previously detailed by Tiwari et al. (2006). 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 (Finnzymes Oy, Espoo, Finland) 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 s), annealing at 56 °C (for 30 s), and extension at 72 °C (for 30 s), followed by a final extension at 72 °C for 5 min. After amplification, 1 μl TaaI restriction endonuclease (5 U/μl; Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA), and 1.22 μl 10 × digestion buffer were added to the total PCR volume. Digestion was carried out at 65 °C, overnight. Digested fragments were resolved on 4% agarose gel stained with GRGreen Nucleic Acid Gel Stain and visualized under UV light. Genotype determination was based on the fragment lengths such as 305 bp for S allele and 257 bp for R allele.

2.3. Statistical methods

In this survey, the nomenclature of genotype and allele frequency, we followed, were detailed by Pierce (2012). The proportions were determined with 95% confidence interval (CI95%) (Reiczigel et al., 2010). For comparison of genotype and allele frequency in the ruminant populations, chi-square test with significance level of 0.05 was performed with Bonferroni correction using R statistical software i386 3.3.0 version.

3. Results

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 hosts (Table 1). Difference of allele frequencies between the host populations was confirmed significant by chi-square test.

4. Discussion

Although several studies were concerned with AR in small ruminants (Papadopoulos et al., 2012; Rose et al., 2015), our knowledge is very deficient in connection with wild ruminants (Chintoan-Uta et al., 2014; Mackintosh et al., 2014) We investigated the BZ resistance status of *H. contortus,* collected from sheep and two sympatric deer species and our results confirmed considerable divergence between the hosts. In Hungarian veterinary practice, one of the most preferred drug groups is BZs; therefore, their usage in parasite management is considered very general. Our results suggest that the high level of R allele proportion in sheep in this region is derived from the high treatment frequency and the long-term unrotated drug application; as our previous work confirmed it (under publication). On the other hand, in Hungary, the usage of anthelmintics and other prescription medicines for hunting parties are restricted by the food safety law, because the consumption of the therapeutic dose cannot be ensured and the withdrawal period cannot be controlled in wildlife. In consequence, the hunting parties of the studied area have never applied any anthelmintics. Based on these