Annals of Parasitology 2016, 62(2), 119–123
doi: 10.17420/ap6202.43

Original papers

Benzimidazole resistance in the ovine *Haemonchus contortus* from southern Poland – coproscopical and molecular findings

Jerzy Kowal[1], Anna Wyrobisz[1], Paweł Nosal[1], Mirosław Kucharski[2], Urszula Kaczor[2], Marta Skalska[1], Piotr Sendor[3,4]

[1]Institute of Animal Sciences, University of Agriculture in Kraków, al. Mickiewicza 24/28, 30-059 Kraków; Poland
[2]Department of Animal Biotechnology, University of Agriculture in Krakow, ul. Rędzina 1b, 30-248 Kraków; Poland
[3]Małopolska Agricultural Advisory Centre in Karniowice, os. XXXV-lecia PRL 9, 32-082 Bolechowice; Poland
[4]Department Of Technology, Ecology and Economics of Animal Production, The National Research Institute of Animal Production, ul. Krakowska 1, 32-083 Balice; Poland

Corresponding Author: Anna Wyrobisz; e-mail: a.wyrobisz@gmail.com

ABSTRACT. Anthelmintic resistance within nematodes has become a very common issue, however, the data about its occurrence in the gastrointestinal nematodes of sheep in Poland are very limited. This study was carried out in order to evaluate the presence of benzimidazole resistance in highly pathogenic *Haemonchus contortus* by means of parasitological and molecular techniques. The research represents the first Polish attempt to confirm the presence of a mutation at codon 200 of the β-tubulin isotype 1 gene in *H. contortus* by PCR-RFLP. The occurrence of this mutation indicates the risk of ineffective benzimidazole treatment, nowadays commonly used for parasite control in Poland. The resistant parasites were detected by means of FECRT in a sheep flock (30 individuals) from southern Poland. To confirm the resistance on the molecular level, primers designed according to the sequences available in Genebank were used to detect the mutation. Unfortunately, as the PCR product was shorter than required (403 bp), further analyses are needed. The obtained results may indicate the high variability within the parasite population. Hence, it is essential to adapt the reaction conditions to our geographic strain of the nematode – and further analyses are required.

Key words: *Haemonchus contortus*, benzimidazole resistance, PCR, Poland

Introduction

Effective control and proper treatment of parasitoses, especially those caused by the presence of gastrointestinal nematodes (GIN) in ruminants, is not always possible due to the presence of resistant parasitic strains. This phenomenon is defined as an increase in frequency of individuals able to tolerate elevated drug doses relative to a normal population [1]. Anthelmintic resistance (AR) has become a significant problem among grazing livestock, and threatens both agricultural income and animal welfare. More seriously, multiple drug-resistant populations have also been detected [2–5].

The most common resistant genera are: *Haemonchus*, *Teladorsagia* and *Trichostrongylus* [6]. Although many bases exist for the acquisition of AR, recent reports make particular reference to molecular ones. *Haemonchus contortus* is the most pathogenic and prevalent species of the abomasal nematodes in small ruminants. This haematophagous parasite causes production losses and even death in severe cases. Nowadays, three broad-spectrum anthelmintic groups available for haemonchosis treatment in grazing animals are commonly used: benzimidazoles (BZs), imidazothiazoles and macrocyclic lactones. Among these, BZs are preferred for control of strongyle infections due to their high efficiency (>95%), and absence of toxic residues in milk or meat [7,8].

BZs lead to inhibition of the microtubule formation by binding selectively to the cellular β-tubulin of the helminth [9]. In *H. contortus*, this binding is prevented by a point mutation in the β-tubulin gene; several reports have implicated the presence of Single Nucleotide Polymorphisms

(SNPs) at codons 167, 198 or 200 of the β-tubulin isotype 1 gene in the acquisition of BZ resistance [10–13]. However, the SNP at codon 200 (TTC to TAC) is the most important mutation linked with such resistance in *H. contortus*, resulting in substitution of phenylalanine (Phe) for tyrosine (Tyr) [12,14–17].

Despite its great veterinary and economical significance, data concerning the occurrence of AR in GIN of sheep in Poland are very limited [18,19]. Furthermore, information on molecular assays for BZ-resistance are not available. Therefore, the purpose of the presented study was to confirm the presence of BZ-resistance, acquired through the F200Y polymorphism, in a highly pathogenic *H. contortus* in sheep in Poland by PCR-RFLP.

Materials and Methods

The study involved 30 adult Polish Mountain sheep from southern Poland. The flock was created to graze on the embankments in the rural areas near Kraków, as a part of the owcenawaly.pl research project. Animals used in the trial were chosen according to Coles et al. [17]. During the project, the sheep were dewormed with Valbazen 10% (Pfizer; 5 mg/kg body weight; *per os*, single dose – according to the manufacturer's recommendations).

Faecal egg count reduction test (FECRT). BZ resistance was ascertained by a combination of appropriate parasitological methods. The fecal egg count reduction test (FECRT) was applied to assess the efficacy of the used compound. The concentration McMaster technique [20] was used to detect helminth eggs in the faecal samples taken individually from each animal. Furthermore, FECRT was supported by the identification of larvae obtained from the coproculture [17]. The larval cultures (pre- and posttreatment) were established in accordance with the method proposed by Henriksen and Korsholm [21]. The harvested third-stage larvae were subsequently distinguished according to van Wyk and Mayhew [22]. The FECRs were calculated only for sheep that excreted more than 150 eggs per gram of faeces (EPG) at the beginning of treatment (Day 0), as suggested for adult sheep [17]. Since no control group was used, the formula: (pretreatment FEC – posttreatment FEC/pretreatment FEC) × 100 was applied, and the mean value was calculated.

Table 1. Efficacy of albendazole against *Haemonchus contortus* using FECRT

No. of sheep	Day 0 [EPG]	Day 10 [EPG]	FECR [%]
1	160	0	100
2	280	0	100
3	160	0	100
4	300	0	100
5	2100	2100	0,0
6	220	40	81.8
7	160	20	87.5
8	160	20	87.5
mean FECR [%]			38.1

Sample collection and DNA extraction. Nematodes were collected directly from the abomasum during the *post-mortem* examination of four inefficiently treated sheep. Species identification was performed by a study of characteristic morphological features [23]. In total, 55 specimens of *H. contortus* were collected and then stored in 70% ethanol for further analyses. For the DNA extraction, only the anterior part of the female body and adult males were used to avoid the possibility of the samples being contaminated with DNA from the eggs. Subsequently, nematodes were removed from ethanol, washed and cut into small pieces in the saline solution. DNA extraction was performed from a single nematode using a DNA extraction kit (Promega) according to the manufacturer's instructions; however, Proteinase K and ß-mercaptoethanol were added to achieve a significantly higher DNA concentration. All extracted DNA samples were properly stored until the first step of the molecular analysis.

Polymerase Chain Reaction (PCR). The standard Polymerase Chain Reaction method (PCR) was carried out to amplify the part of isotype 1 β-tubulin gene containing the codon 200 triplet, with commonly used primers designed according to the sequences available in Genebank: the forward primer (P1) from nucleotide in position 446 to 467 (22-mer) and the reverse primer (P2) between nucleotides 825 to 847 (23-mer) [24,25]. The volume and content of the PCR reaction mixture as well as the PCR program itself (Eppendorf Gradient S thermocycler, Germany) were applied as previously described [24]. Samples without genomic DNA were used as negative controls.

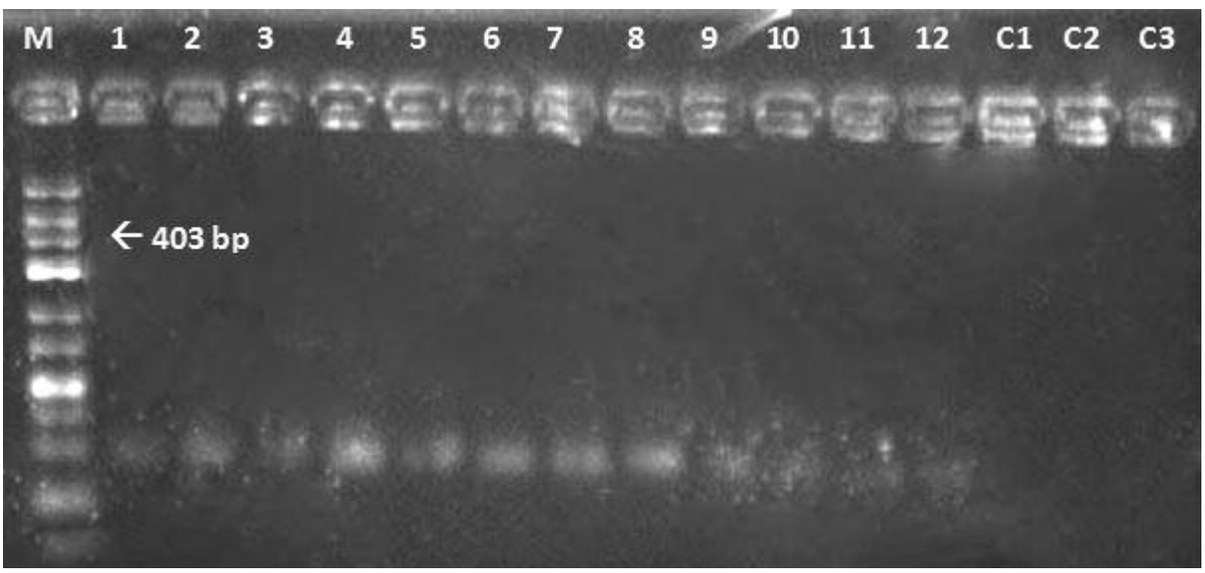

Fig. 1. Ethidium bromide-stained agarose gel electrophoresis of PCR products generated from genomic DNA of *Haemonchus contortus* (part of analyzed samples). **M,** Gene Ruler Low Range DNA Ladder, ready-to-use (ThermoFischerScientific, USA). **1-12**, samples with genomic DNA from *H. contortus*. **C1-C3**, control samples.

Results

Faecal egg count reduction test (FECRT)

The results obtained with the FECRT indicated a low level of GIN infection. The outcomes varied from 10 to 2100 EPG before drug application (Day 0), and from 20 to 2100 EPG after the 10-day-treatment (Day 10). The resistance was stated for *Haemonchus contortus*: only larvae of this species were obtained from posttreatment coprocultures. The FECR value evaluated for this species was low and reached an average of 38.1%, ranging from 0 to 100% for individual sheep (Table 1) (95% confidence interval; range 53.5–110.2%). The other species of GIN (i.e., *Trichostrongylus* sp., *Teladorsagia* sp. and *Chabertia ovina*), as well as lung nematode (i.e., *Dictyocaulus filaria*, *Muellerius capilaris*), demonstrated high susceptibility to the drug.

Polymerase Chain Reaction (PCR)

The PCR products were analyzed on 2% agarose gel in 0.5× TBE stained with ethidium bromide and visualized using an UV illuminator. Regrettably, as the amplified fragment was shorter than expected 403 bp (less than 100 bp – Fig. 1), the complete PCR-RFLP method could not be performed. This fragment was also not long enough for effective sequencing, therefore it was impossible to verify whether the amplified product was an isotype 1 β-tubulin gene, or confirm which part of this gene had been obtained.

Discussion

The presence of AR in GIN nematodes has been noted worldwide [6]. There is hence a need to optimize the use of anthelmintics and to reduce the risk of further enhancement of AR. Although it has been shown that both *in vivo* and *in vitro* methods are comparable to molecular tests with regard to their analytical ability, they appear to be less sensitive [26], and more importantly, cannot provide the information on the diverse resistance mechanisms needed to monitor the spread of AR.

The occurrence of AR in Europe is well documented especially in Western European, as well as Scandinavian countries. The majority of studies address the species of the three most important genera of sheep nematodes, *Haemonchus*, *Teladorsagia* and *Trichostrongylus*, and show BZ-resistance as a very serious case. For instance, trichostrongylid nematodes resistant to BZs have been identified in the United Kingdom, Spain, France, Greece and the Netherlands. Furthermore, *H. contortus* has been shown to be the predominant resistant or even multi-resistant species in a number of countries [2,6].

On the other hand, data on the issue in Poland, as well as in our neighboring countries are very limited. Research conducted in the Czech Republic [27], Slovakia [28,29], or Lithuania [30] have addresses the topics of BZ-resistance in GIN, but only using *in vitro* and *in vivo* methods. Likewise,

Polish analyses refer only to the coproscopical approaches [18,19].

Various molecular methods are applicable for the detection of BZ-resistance caused by SNP at codon 200. The most common is PCR-RFLP and its variations [12,24,31]; however, allele-specific PCR (AS-PCR) [17,32], real-time PCR (qPCR) [33], single-strand conformation polymorphism (SSCP) analysis, or pyrosequencing [34,35] can also be used.

As, this is the only Polish attempt to confirm the presence of a mutation at codon 200 of the β-tubulin isotype 1 gene in the literature, our findings cannot be properly compared. The obtained results reflect the high variability within the parasite population. Hence, it is essential to adapt the reaction conditions to the local geographic strain of the nematode, including the design of primers used to amplify the DNA marker. To date, *H. contortus* is one of the most intensively studied species and while its AR has been documented as widespread, it is not sufficiently recognized in Poland. Therefore, further studies are required to validate the species-specific genetic markers. Although the results of the molecular test were not consistent with those of parasitological examinations, our findings confirm the presence of BZ-resistance in *H. contortus*, which is known to be one of the most pathogenic species in Polish sheep flocks.

Acknowledgements

This research was financed by the Ministry of Science and Higher Education of the Republic of Poland.

References

[1] Prichard R.K., Hall C.A., Kelley J.D., Martin I.C.A., Donald A.D. 1980. The problem of anthelmintic resistance in nematodes. *Australian Veterinary Journal* 56:239-251.

[2] Bartley D.J., Jackson F., Jackson E., Sargison N. 2004. Characterisation of two triple resistant field isolates of *Teladorsagia* from Scottish lowland sheep farms. *Veterinary Parasitology* 123:189-199.

[3] Sargison N.D., Jackson F., Bartley D.J., Moir A.C. 2005. Failure of moxidectin to control benzimidazole-, levamisole- and ivermectin-resistant *Teladorsagia circumcincta* in a sheep flock. *Veterinary Record* 156:105-109.

[4] Traversa D., Paoletti B., Otranto D., Miller J. 2007. First report of multiple drug resistance in trichostrongyles affecting sheep under field conditions in Italy. *Parasitology Research* 101:1713-1716.

[5] Höglund J., Gustafsson K., Ljungstrom B.L., Engstrom A., Donnan A., Skuce P. 2009. Anthelmintic resistance in Swedish sheep flocks based on a comparison of the results from the faecal egg count reduction test and resistant allele frequencies of the ß-tubulin gene. *Veterinary Parasitology* 161:60-68.

[6] Papadopoulos E., Gallidis E., Ptochos S. 2012. Anthelmintic resistance in sheep in Europe: A selected review. *Veterinary Parasitology* 189:85-88.

[7] Dorny P., Romjali E., Feldman K., Barubara A., Pandey V.C. 1995. Studies on the efficacy of four anthelmintics against strongyle infections of sheep in North Sumatra, Indonesia. *Asian-Australian Journal of Animal Science* 8:347-352.

[8] Kumar P., Malik S., Kumar N., Mehra R., Bhunyan M. 2012. Acute and subacute toxicity studies of Mannich bases of Benzimidazole derivatives. *International Journal of Pharmacy and Pharmaceutical Sciences* 1:94-110.

[9] Lacey E. 1988. The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. *International Journal for Parasitology* 7:41-48.

[10] Silvestre A., Humbert J.F. 2002. Diversity of benzimidazole-resistance alleles in populations of small ruminants parasites. *International Journal for Parasitology* 32:921-928.

[11] Samson-Himmelstsjerna G.N. 2006. Molecular diagnosis of anthelmintic resistance. *Veterinary Parasitology* 136:99-107.

[12] Ghisi M., Kaminsky R., Maser P. 2007. Phenotyping and genotyping of *H. contortus* isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. *Veterinary Parasitology* 144:313-320.

[13] Rufener L., Kaminsky R., Maser P. 2009. In vitro selection of *H. contortus* for benzimidazole resistance reveals a mutation at amino acid 198 of β-tubulin. *Molecular and Biochemical Parasitology* 168:120-122.

[14] Kwa M.S., Veenstra J.G., Roos M.H. 1994. Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. *Molecular and Biochemical Parasitology* 63:299-303.

[15] Elard L., Comes A.M., Humbert J.F. 1996. Sequences of b-tubulin cDNA from benzimidazole-susceptible and -resistant strains of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. *Molecular and Biochemical Parasitology* 79:249-253.

[16] Kaplan R.M. 2004. Drug resistance in nematodes of veterinary importance: a status report. *Trends in*

Parasitology 20:477-481.
[17] 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. *Veterinary Parasitology* 136:167-185.
[18] Balicka-Ramisz A., Ramisz A., Malecki J., Supera K. 1997. Przypadek lekooporności nicieni żołądkowo-jelitowych na fenbendazol u owiec. *Magazyn Weterynaryjny* 6:442-443.
[19] Balicka-Ramisz A., Ramisz A. 1999. Benzimidazol resistance in nematode parasites in domesticated animals in north-west part Poland, *EJPAU Animal Husbandry*:2.
[20] Roepstorff A., Nansen P. 1998. Epidemiology, diagnosis and control of helminth parasites of swine. FAO, Rome.
[21] Henriksen S.A., Korsholm H. 1983. A method for culture and recovery of gastrointestinal strongyle larvae. *Nordisk Veterinaer Medicin* 35:429-430.
[22] van Wyk J.A., Cabaret J., Michael L.M. 2004. Morphological identification of nematode larvae of small ruminants and cattle simplified. *Veterinary Parasitology* 119:277-306.
[23] 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 of North America. *Journal of Parasitology* 80:107-119.
[24] Nabavi R., Shayan P., Shokrani H.R., Eslami A., Bokaie S. 2011. Evaluation of benzimidazole resistance in *Haemonchus contortus* using comparative PCR-RFLP methods. *Iranian Journal of Parasitology* 6:45-53.
[25] Kwa S.G., Veenstra J.G., Roos M.H. 1993. Molecular characterization of beta-tubulin genes present in benzimidazole resistant populations of *Haemonchus contortus. Molecular and Biochemical Parasitology* 63:133-144.
[26] Demeler J., Krüger N., Krücken J., von der Heydel V.C., Ramünke S., Küttler U., Miltsch S., López Cepeda M., Knox M., Vercruysse J., Geldhof P., Harder A., von Samson-Himmelstjerna G. 2013. Phylogenetic characterization of beta-tubulins and development of pyrosequencing assays for benzimidazole resistance in cattle nematodes. *PloS One* 8:e70212.
[27] Vernerová E., Vondrová R., Kisová H., Svobodová V., Hera A. 2009. Detection of benzimidazole resistance in gastrointestinal nematode parasites of sheep in the Czech Republic. *Veterinarni Medicina* 54: 467-472.
[28] Čerňanská D., Várady M., Čorba J. 2006. A survey on anthelmintic resistance in nematode parasites of sheep in the Slovak Republic. *Veterinary Parasitology* 15:39-45.
[29] Dolinska M., Ivanišinova O., Königová A., Várady M. 2014. Anthelmintic resistance in sheep gastrointestinal nematodes in Slovakia detected by in-vitro methods. *BMC Veterinary Research* 10:233.
[30] Kupčinskas T., Stadalienė I., Šarkūnas M., Riškevičienė V., Várady M., Höglund J., Petkevičius S. 2015. Prevalence of anthelmintic resistance on Lithuanian sheep farms assessed by in vitro methods. *Acta Veterinaria Scandinavica* 57:88.
[31] Shokrani H.R., Shayan P., Eslami A., Nabavi R. 2012. Benzimidazole resistance in *Haemonchus contortus*: new PCR-RFLP method for the detection of point mutation At codon 167 of isotype 1 ß-tubulin gene. *Iranian Journal of Parasitology* 7:41-48.
[32] Rufener L., Kaminsky R., Maser P. 2009. In vitro selection of *Haemonchus contortus* for benzimidazole resistance reveals a mutation at amino acid 198 of ß-tubulin. *Molecular and Biochemical Parasitology* 168:120-122.
[33] Alvarez-Sanchez M.A., Perez-Garcia J., Cruz-Rojo M.A., Rojo-Vazquez F.A. 2005. Real time PCR for the diagnosis of benzimidazole resistance in trichostrongylids of sheep. *Veterinary Parasitology* 129:291-298.
[34] Skuce P., Stenhouse L., Jackson F., Hypsa V., Gilleard J. 2010. Benzimidazole resistance allele haplotype diversity in United Kingdom isolates of *Teladorsagia circumcincta* supports' a hypothesis of multiple origins of resistance by recurrent mutation. *International Journal of Parasitology* 40:1247-1255.
[35] Knapp-Lawitzke F., Krücken J., Ramünke S., von Samson-Himmelstjerna G., Demeler J. 2015. Rapid selection for ß-tubulin alleles in codon 200 conferring benzimidazole resistance in an *Ostertagia ostertagi* isolate on pasture. *Veterinary Parasitology* 209:84-92.

Received 2 February 2016
Accepted 18 April 2016