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Original article

Development of resistance to eprinomectin in gastrointestinal nematodes in a goat herd with pre-existing resistance to benzimidazoles

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Abstract

We described a first case of resistance to eprinomectin in goat herd in Poland in which resistance to benzimidazoles had been previously reported. The herd was established in 2011 by purchasing several goats from a single herd in south-eastern Poland. Resistance to benzimidazoles in the herd was first reported in 2017. Shortly after the owner started to signal low effectiveness of the treatment with eprinomectin. In June 2018 the larval development test from pooled faecal sample was performed and the results indicated the presence of resistance to macrocyclic lactones and levamisole. In July 2018 a faecal egg count (FEC) reduction test was performed in 39 animals with levamisole, eprinomectin and one untreated control group. Drugs were used in doses recommended for goats. Three methods of calculation of FEC reduction were compared. After eprinomectin treatment, FEC reduction ranged from 0 to 20%, depending on the method of calculation. FEC reduction following levamisole treatment was 100%.

Main species present in the faecal samples after treatment and in larvicidal concentrations in larval development test was *Haemonchus contortus*. This is the first report of anthelminthic resistance to macrocylic lactones (eprinomectin) in goats in Poland.

Key words: anthelmintic resistance, eprinomectin, levamisole, faecal egg count reduction test, larval development test, *Haemonchus contortus*, *Trichostrongylus* spp., *Teladorsagia* spp., goats

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Introduction

Gastrointestinal nematodes cause the most prevalent and economically important diseases of small ruminants. They are responsible for both direct and indirect major losses generated by decreased productivity, costs of control measures and death of animals. Therefore, their effective control is essential. Unfortunately, constant use of anthelmintics without any preceding testing is the mainstay of prevention in many herds. Practiced worldwide for last three decades, this approach has led to the emergence of anthelmintic resistance (AR) in gastrointestinal nematodes which is now the main threat to goat farming. AR is a genetic change in the ability of parasites to survive treatments with recommended doses of anthelmintic (Taylor and Hunt 1989). The term 'resistance' describes the condition of nematode populations that, despite being previously sensitive to anthelmintics, inherit the ability to survive and evade the toxic effects of drugs after repeated administration (Várady et al. 2011). Numerous factors such as under-dosing, using the same group of anthelmintics for a long time, frequently repeated treatments, or lack of quarantine are responsible for accelerating the development of AR (Kaplan 2004, Hoste et al. 2008). AR in goats has so far been reported in several European countries including the Great Britain (Jackson et al. 1992, Hong et al. 1996), the Netherlands (Borgsteede et al. 1996), Spain (Requejo-Fernández et al. 1997), Italy (Cringoli et al. 2007), France (Paraud et al. 2009), Germany (Bauer 2001), Switzerland (Schnyder et al. 2005), Denmark (Peña-Espinoza et al. 2014), Norway (Domke et al. 2012) and Slovakia (Babják et al. 2018). In Poland, drug resistance of gastrointestinal nematodes was found and described in sheep (Balicka-Ramisz and Ramisz 1999, Kowal et al. 2016), cattle, horses and pigs (Balicka-Ramisz and Ramisz 1999), but these cases were confirmed only by faecal egg count reduction test (FECRT). In goats, the first case of resistance to albendazole was reported by authors of this study in 2017 (Mickiewicz et al. 2017). Moreover, this case concerned the same herd which was re-examined in terms of levamisole (LEV) and eprinomectin (EPM) effectiveness in the study. Data on the prevalence and spread of AR of gastrointestinal nematodes in Poland are still insufficient leading to the wrong conclusion that this problem is absent in Poland, in spite of the fact that all of the above mentioned factors accelerating the development of AR are commonly found in goat herds' management practices. The aim of this study was to investigate the presence of the resistance to anthelmintics from macrocyclic lactones group in goats in Poland, and examine the level of decrease in effectiveness of eprinomectin after two years of intensive use in the herd with previously reported resistance to benzimidazoles.

Materials and Methods

Study herd

The presence of the resistance of gastrointestinal nematodes to benzimidazoles (albendazole) was previously described on this farm in 2017 by Mickiewicz et al. (2017). The herd consisted of 2 adult males, 53 adult females and 23 kids of Polish White Improved and Polish Fawn Improved breeds. Goats were kept in the half-wooden-half-brick barn. The animals were grazed from April to November for 8 to 10 hours per day on 5.65 ha pasture. The pasture bordered on a pasture used by a 200-head sheep flock. Although both were separated by an electric-wire fence, goats used to cross it and graze with sheep. The faecal egg counts (FECs) in the goat herd had been performed monthly since September 2017, using the modified McMaster method (Coles et al. 1992) and macrocyclic lactones (eprinomectin, ivermectin) had been used for anthelmintic treatment of the herd since 2015. In May 2016 first faecal egg count reduction test (FECRT) with eprinomectin (1 mg/kg pour on, Eprizero®, Poland, 5mg/ml pour on, SCANVET, Poland), was performed on 56 adult goats without a control group and the percentage of FEC reduction (FECR%) 14 days after treatment was 97%. The average number of anthelmintic treatments was of 4 to 5 per year, depending on the occurrence of clinical signs of parasitism and FEC (Table 1). Additionally, the sheep flock were dewormed by the owner twice a year only with ivermectin. In July 2018 the owner claimed that the last anthelmintic treatment with eprinomectin was ineffective.

Faecal egg count reduction test (FECRT)

In July 2018 FEC performed in 48 animals from the herd revealed a mean egg count of 1031 (SD ±1506) EPG. From this group 39 goats with minimum FEC of 150 EPG were selected. All selected animals were older than 6 months and they had not been treated with any anthelmintics for at least 8 weeks prior to the study. During the study, animals were kept only in the barn.

The FECRT was performed according to the guidelines of the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) described by Coles et al. (1992, 2006). Animals were randomly divided into three equal groups (n=13), two for treatment and one as a control. Prior to the treatment,

Table 1. The history of anthelmintic treatment of the herd.

Data	Anthelmintic	Dosage
July 2018 (FECRT)	eprinomectin (Eprinex®)	1 mg/kg pour on
July 2018 (FECRT)	levamisole (Levamol®)	12 mg/kg p.o.
March 2018	albendazole (Valbazen®)	20 mg/kg p.o.
March 2018	ivermectin (Biomectin®)	0,4 mg/kg s.c.
January2018	ivermectin (Biomectin®)	0,4 mg/kg s.c.
December 2017	eprinomectin (Eprinex®)	1 mg/kg pour on
September 2017	albendazole (Valbazen®)	20 mg/kg p.o.
July 2017	eprinomectin (Eprizero®)	1 mg/kg pour on
May 2017	albendazole (Valbazen®)	15 mg/kg p.o.
February 2017	eprinomectin (Eprizero®)	1 mg/kg pour on
August 2016	albendazole (Valbazen®)	15 mg/kg p.o.
May 2016 (FECRT)	eprinomectin (Eprizero®)	2ml/10 kg
November 2015	albendazole (Valbazen®)	5 mg/kg p.o.
May 2015	ivermectin (Biomectin®)	0.1 mg/kg s.c.
2011 – 2014	fenbendazole (Fenbenat®)	200 mg/goat independent on weight and repeating of treatment after 14 days

animals were weighed individually on an electronic scale. Goats were treated with recommended doses of levamisole (12 mg/kg p. o., Levamol® oral powder 80 mg/g, VETOQUINOL, Poland; LEV) and eprinomectin (1 mg/kg pour on, Eprizero® , 5mg/ml pour on, SCANVET, Poland; EPM). The medicines were given by the owner. The control group remained untreated for the entire study period. All anthelmintics used in this study were given in extra label pattern, at time of the study none of these anthelmintics was registered in Poland for their use in goats. Faecal samples were collected from the rectum of the animals on the day of the treatment (day 0), and on day 5 and 14 after the treatment, packed in sealed bags and delivered to the laboratory. Samples were examined within 24 hours after collection by modified McMaster technique with a sensitivity of 50 EPG according to W.A.A.V.P. guidelines (Coles et al. 1992, 2006). Larval cultures were prepared for each group by mixing 5 g of faeces collected from each animal on day 0 and on day 14 into one pool per group. After baermannization, a minimum of 100 third stage larvae (L3) from each pool were identified at the genus/species level following the procedure described by van Wyk and Mayhew (2014), and the percentage of each nematode genus/species present was calculated. Differentiation between *Trichostrongylus* spp. and *Teladorsagia* spp. was performed after exsheathement of L3 larvae in sodium hypochlorite solution for 5 minutes in a Petri dish containing 4 ml larvae suspension and 180 ml sodium hypochlorite (aqueous solution, about 3.5% active Cl, rectapur, PROLABO, Singapore). Subsequently, L3 larvae differentiation was performed under 400x light microscope magnification (Nikon Eclipse E200), by comparing specific morphological features of each L3 species (McMurtry et al. 2000).

Larval development test (LDT)

The larval development test was performed in June 2018 at the Institute of Parasitology of Slovak Academy of Sciences in Kosice from anaerobically stored pooled faecal samples. Fresh faecal samples were collected from 15-20 randomly selected adult goats from the herd, pooled, homogenized in tap water and used to completely fill 100 ml bottles to provide anaerobic conditions according to the method described by Hunt and Taylor (1989). Samples were delivered to the laboratory and proceeded within 48 hours after collection. Eggs were extracted from samples by sieving in 250, 100 and 25µm sieves, centrifugation, and flotation in Sheather's sugar solution. Then, eggs were inspected microscopically to ensure that embryonation had not yet begun and suspended in deionized water at a concentration 70-100 eggs per l0 µl. Stock solutions of thiabendazole (SIGMA-ALDRICH, MERC, Germany), levamisole (SIGMA-ALDRICH, MERC, Germany) and ivermectin aglycone (TEBU-BIO, France; IVM-AG) were prepared by dissolving pure drugs in dimethyl sulfoxide (DMSO; SIGMA--ALDRICH, MERC, Germany) and serially diluted 1:2 in DMSO (thiabendazole, ivermectin-aglycone) or in deionised water (levamisole) to produce 12 final concentrations of thiabendazole, ivermectin aglycone and levamisole ranged from 0.0006 to 1.28 µg/ml, from 0.084 to 173.6 ng/ml ivermectin aglycone, and from 0.020 to 32 µg/ml thiabendazole, respectively. As it was demonstrated by Dolinská et al. (2012, 2013) the use

Table 2. Faecal egg count reduction test results.

	Control	LEV	EPM
FEC day 0 p. t. (± SD)	992 (±200)	1500 (±2513)	1208 (±268)
FEC day 5 and 14 for LEV and day 14 for EPM p. t. (± SD)	739 (±553) 962 (±194)	0	965 (±320)
[1]FECR%	-	100	0
[95% CI]	-	100-100	(-122)-50
[2]FECR%	3	100	20
[3]FECR%	-	100	17

of ivermectin aglycone significantly increased capacity of the test to distinguish between ivermectin resistant and susceptible strains of *Haemonchus contortus*. Tests were performed according to the procedure described by Hubert and Kerboeuf (1992) with further modifications of Várady et al. (1996). Tests were performed in 96 wells cell culture plates (SARSTED, Germany) and culture medium comprised 10 µl of either (all in one test) thiabendazole, ivermectin-aglycone, levamisole or DMSO (control wells) solution, 110 µl of deionised water, 20 ml of culture medium as described by Hubert and Kerboeuf (1984) and 10 ml of a suspension (approximately 70-100 eggs) containing Amphotericin B (SIGMA-ALDRICH, MERCK, Germany) at a concentration of 5 mg/ml. Tests were performed using two replicates at each drug concentration. The plates were sealed to prevent drying and incubated for 7 days at 27°C. After the incubation period, 10 µl od Lugol's solution were added to each well to stop larval development. After incubation, the proportion of unhatched eggs and L1-L3 larvae in each well were counted under an inverted microscope. The L3 larvae in both tested and control wells were identified at the genus/species level following the procedure described by van Wyk and Mayhew (2014).

Statistical analysis

Faecal egg count reduction percentages (FECR%), were calculated comparing three different methods:

1. FECR% = 100% × (1 –T_2/C_2), according to Coles et al. (1992, 2006)
2. FECR% = 100% × (1 – T_2/T_1), according to Kochapakdee et al. (1995)
3. FECR% = 100% × (1 − [T2/T1] × [C1/C2]) according to Dash et al. (1988)

Faecal egg count arithmetic mean on day 0 and day 14 in treated group are indicated as T_1 and T_2, and C_1 and C2 is arithmetic mean of FEC of the control group on day 0 and 14 after treatment. Anthelmintic resistance is present when FECR% is less than 95% (Kochapakdee et al. 1995) and the 95% lower confidence level (95% CI) is less than 90% (Coles et al. 1992, 2006). If only one of these conditions is fulfilled, anthelmintic resistance is suspected in the method proposed by Coles et al. (1992, 2006). For the method by Dash et al. (1988), anthelmintic resistance is present if FECR% is less than 80%.

Results of LDT are presented as LC_{50}/LC_{99} (lethal concentration; LC) estimates, which are defined as the anthelmintic concentration of thiabendazole, levamisole and ivermectin aglycone where development to the L3 stage is inhibited by 50% and 99%, respectively. The data were analysed by a logistic regression model to determine LC_{50}/LC_{99} (Dobson et al. 1987).

Discrimination doses for thiabendazole, levamisole and ivermectin aglycone used in LDT were established as 0.02 µg/ml, 0.5 µg/ml (Coles et al. 2006) and 21.6 ng/ml (Dolinská et al. 2014), respectively.

Results

Faecal egg count reduction test

The results of FECRT are presented in detail in Table 2.

FEC reduction in the untreated control group was only 3% during the study period. Treatment with eprinomectin reduced the FEC between 0% (95% CI: -122% to 55%), 20% and 17% (depending on the method used). Individual FEC at day 0 and 14 are presented in detail in Table 3. Resistance was identified by all methods applied.

Larval culture prepared from the faecal samples collected on day 14 consisted of *H. contortus* 82%, *T. circumcincta* 9%, *T. colubriformis* 9% in the group treated with eprinomectin and of *H. contortus* 98%, *T. circumcincta* 1%, *T. colubriformis* 1% in the untreated control group

FEC reduction after treatment with levamisole was significant and was 100% in all of used calculation methods indicating that the gastrointestinal nematodes were fully susceptible to this drug. Individual FEC on day 0 and 5 are presented in detail in Table 3. Although no eggs of gastrointestinal nematodes were found by modified McMaster method used in FECRT, a small number of *T. circumcincta* larvae were found in faecal cultures prepared from samples collected on day 5 and 14.

Table 3. Individual faecal egg counts (eggs per gram; EPG) at day 0, 5 and 14.

Animal	EPG day 0	EPG day 5	EPG day 14
1. C	2500	1250	1150
2. C	2150	1550	2050
3. C	1650	900	1250
4. C	1250	600	550
5. C	1050	1900	2200
6. C	900	550	1450
7. C	750	950	1550
8. C	750	600	850
9. C	550	0	350
10. C	450	400	350
11. C	400	300	300
12. C	400	400	300
13. C	150	200	150
14. LEV	9700	0	-
15. LEV	600	0	-
16. LEV	1900	0	-
17. LEV	1500	0	-
18. LEV	1200	0	-
19. LEV	1050	0	-
20. LEV	800	0	-
21. LEV	750	0	-
22. LEV	700	0	-
23. LEV	500	0	-
24. LEV	400	0	-
25. LEV	250	0	-
26. LEV	150	0	-
27. EPM	3500	-	2700
28. EPM	2150	-	3750
29. EPM	1750	-	1000
30. EPM	1250	-	0
31. EPM	1150	-	550
32. EPM	950	-	950
33. EPM	750	-	0
34. EPM	750	-	1150
35. EPM	2200	-	1750
36. EPM	500	-	200
37. EPM	400	-	250
38. EPM	200	-	150
39. EPM	150	-	100

* C – untreated control group
* LEV – levamisole treated group
* EPM – eprinomectin treated group

Larval development test

Larval development was observed in all wells, also in those where the concentrations of each tested antihelmintic is considered larvicidal. The LC_{50} values for thiabendazole, ivermectin aglycone and levamisole were 1.29 µg/ml, 178.62 ng/ml, and 0.94 µg/ml, respectively. Resistance to all tested anthelmintics was observed. The composition of nematodes species present in wells with the highest concentrations of thiabenda-

zole (1.28 µg/ml), ivermectin aglycone (173.6 ng/ml), and levamisole (32 µg/ml) where L3 larvae development occur were *H. contortus*; *H. contortus, Trichostrongylus* spp., and *Teladorsagia* spp.; *H. contortus and Teladorsagia spp.,* respectively. In the control wells, the composition of L3 larvae genus/species was *H. contortus, Trichostrongylus* spp. *and Teladorsagia* spp.

Discussion

The presented results of reduced faecal egg counts indicated that resistance to eprinomectin in the herd has occurred within a two year period. The value of LC_{50} for ivermectin aglycone in LDT above established discrimination dose also indicated resistance to all drugs from macrocyclic lactones family. Similarly, the LDT results supported the presence of benzimidazoles resistance in the herd previously reported by the authors. In case of levamisole, the LC_{50} was above the established discriminating dose, while FECRT did not confirm the presence of resistance to this drug. In our study discriminating dose of 0.5 µg/ml for levamisole in LDT was established (Coles et al. 2006), although it had been described that the levamisole discriminating dose could differ between the gastrointestinal nematodes species (Coles et al. 1988, Taylor 1990). It was suggested that discriminating doses of 2.5 µg/ml for *H. contortus* (Taylor 1990) and 1.0 µg/ml for *T. colubriformis* (Coles et al. 1988), should be considered for differentiation between susceptible and resistant strains of these species. The presence of small numbers of levamisole resistant alleles in the herd was possible because of the small number of L3 larvae present in both faecal cultures prepared after levamisole treatment and in the LDT, with *Teladorsagia* spp. being the predominant finding. These results may indicate that the herd is at risk of the levamisole resistance development in forthcoming future, which requires constant monitoring of levamisole effectiveness by evaluating the FECR% after every treatment.

In Europe, resistance to macrocyclic lactones in goats has so far been reported only in Denmark (Manigi et al. 1996), Scotland (Jackson et al. 1992), Slovakia (Varady et al. 1993), Switzerland (Schnyder et al. 2005, Artho et al. 2007, Scheuerle et al. 2009, Murri et al. 2014) and Germany (Scheuerle et al. 2009), which suggest that resistance to macrocyclic lactones is not so prevalent in goat popilation in Europe. Our study revealed *H. contortus* as a predominant species present in post-treatment faecal culture, similarly to the findings of studies conducted in Switzerland and Germany where *H. contortus* was suspected to have been imported with goats from Africa (Schnyder et al. 2005, Artho et al. 2007, Scheuerle et al. 2009). However, no goat in our herd was bought from abroad.

According to the results of first FECRT conducted in the herd in 2016 and the history of anthelmintic treatments, we suspect that the resistance to macrocyclic lactones in the herd has developed gradually over several years. In Poland no data on resistance to macrocyclic lactones is available for any animal species. Resistance to benzimidazoles was reported in goats in Poland by the authors of this paper in 2017 for the first time (Mickiewicz et al. 2017).

The predominant gastrointestinal nematode species involved in the past was also *H. contortus*, similarly to findings of this study. The cross-resistance between benzimidazoles and macrocyclic lactones seems to be possible as the same nucleotide changes in ivermectin resistant *H. contortus* have been observed in gene coding for β-tubulin isotype 1 like in benzimidazoles resistant strains (Mottier and Prichard 2008). This could have accelerated development of the macrocyclic lactones resistance in the herd. The pasture on which goats were kept during the grazing season is adjacent to the pasture of large sheep farm, on which, only ivermectin had been used for many years before (no data about dosage and route of administration). As they occasionally shared one pasture, we cannot rule out the transmission of gastrointestinal nematodes with resistant alleles from sheep to goats (Hoste et al. 2010). The owner of the sheep flock refused our offer to perform the FECRT, as well as any other parasitological examinations. Eprinomectin, as one of anthelmintics from macrocyclic lactones group, has the same mechanism of action as ivermectin, so the cross-resistance between these drugs is complete (Lespine et al. 2012).

We have regularly attended this goat herd since 2016, so we are sure that in this time period doses of anthelmintics were appropriate for goats. However, animals were weighted before the treatment only when FECRT were performed. Otherwise, body weight was estimated by the owner and veterinarian without using a scale, which could have resulted in under-dosing. The presence of resistance to benzimidazoles in the herd and easy access to macrocyclic lactone anthelmintics in user-friendly formulations such as pour-on led to almost exclusive use of eprinomectin and (rarely) of ivermectin for the treatment of farm animals. The lesser rotation of groups of anthelmintic compounds used for animal treatments, could have contributed to the development of resistance to macrocyclic lactones in the herd (van Wyk 2001).

Our study is the first report of resistance to macrocyclic lactone anthelmintics in goats in Poland, and the sixth at the country level in Europe. The results of the study indicates that the risk of spread of anthel-

mintic resistance, also multidrug resistance in goats in Poland on increase. Nowadays in Poland, levamisole remained the only available option for the anthelmintic treatment in the examined herd, due to its 100% effectiveness shown by the FECRT. The need for the establishment of parasite control measures based on pasture and farm management techniques with reduction of exclusive reliance upon chemical treatment and prophylaxis will be essential for on farm anthelmintic resistance management in the future.

Based on the results obtained in this study, we conclude that in their daily practice, veterinarians in Poland ought to realize that the multidrug resistance to anthelmintics poses a real danger and individualised treatment schemes should be developed.

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