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Worm-control practices and prevalence of anthelmintic resistance using *in vivo* FECRTs on smallholder sheep farms in Lithuania

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Summary

This study determined the prevalence of anthelmintic resistance (AR) in parasitic nematodes on smallholder sheep farms in Lithuania from April to November 2014. Faecal samples were collected from two groups of 10-15 sheep treated with fenbendazole (FBZ) or ivermectin (IVM) on 18 sheep farms. Two samples were collected from each group: on day zero (T1) and 10-14 days after treatment. Faecal egg counts (eggs per gramme, EPG) were determined using a modified McMaster technique. Animals with <140 EPG on day zero were removed from the analysis. The prevalence of AR was estimated using the *in vivo* faecal egg count reduction test. AR to FBZ was detected on three of 15 farms where FBZ was used (20 %) and was suspected on one farm (6.7 %). AR to IVM was detected on two of 16 farms where IVM was used (12.5 %). The main species of resistant gastrointestinal nematodes (GINs) identified after treatment were *Teladorsagia* spp. and *Trichostrongylus* spp. A questionnaire surveying 71 sheep farmers estimated that 71.8 % of sheep farmers used anthelmintics against GINs. IVM was the most frequently (68.6 %) applied anthelmintic, and 62.7 % of the respondents reported treating their animals twice a year. This study confirmed the presence of AR to GIN infections on sheep farms in Lithuania. Future studies should assess the prevalence of AR to GIN infection using *in vitro* methods.
Keywords: sheep nematodes; anthelmintic resistance; questionnaire survey; FECRT; Lithuania

Introduction

Gastrointestinal nematodes (GINs) of small ruminants have a worldwide distribution (Martinez-Valladares *et al*., 2013). The intensive use of anthelmintics, under-dosing, and repeated treatments with the same anthelmintics have led to the development of resistance, which has become a major and growing problem on sheep and goat farms in many countries (Cernanska *et al*., 2008; Varady *et al*. 2011). The state of anthelmintic resistance (AR) is very problematic in Australia, New Zealand, South Africa, and many Latin American countries (Dolinska *et al*., 2012; Torres-Acosta *et al*., 2012). In Europe, AR has been reported from the Slovak Republic (Cernanska *et al*., 2006), Spain (Alvarez-Sanchez *et al*., 2001), Italy (Traversa *et al*., 2007), Greece (Papadopoulos *et al*., 2001), the United Kingdom (Bartley *et al*., 2006; Taylor *et al*., 2009), and the Netherlands (Borgsteede *et al*., 2007).

The most commonly used anthelmintics belong to three families: macrocyclic lactones (MLs), benzimidazoles (BZs), and imidazothiazoles (Cezar *et al*., 2010). Many reports of AR are cases of BZ or levamisole resistance, but the number of cases of resistance to the ML ivermectin (IVM) is increasing (Papadopoulos, 2008). Reports of resistance to doramectin and moxidectin are less common (Papadopoulos *et al*., 2012). AR can be detected by *in vivo* and *in vitro* methods. The most widely used test to assess anthelmintic efficacy is the *in vivo* faecal egg count reduction test (FECRT), recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Coles *et al*., 1992).

Questionnaire surveys of worm-control practices are very useful for examining the methods of anthelmintic use and animal management (Cernanska *et al*., 2008). Questionnaire surveys have been conducted for sheep farming in England (Fraser *et al*., 2006), Ireland (Patten et at., 2011), and the Slovak Republic (Cernanska *et al*., 2008), for sheep and goats in Denmark (Maingi *et al*., 1996), for goats in northern Italy (Zanzani *et al*., 2014) and in France (Hoste *et al*., 2000).

The aim of this study was to determined the prevalence of anthelmintic resistance and to identify the resistant species of parasitic nematodes on smallholder sheep farms in Lithuania. In addition, to evaluate management and treatment strategy on sheep farms the questionnaire survey was performed.

Materials and methods

Farm questionnaire

A questionnaire surveyed 71 sheep farmers. Twenty-nine farms were visited, and the farmers were interviewed personally. Forty-two sheep farmers from the list of the Lithuanian Sheep Breeders Association were interviewed by telephone. All farmers were asked about their practices of farm management: number of animals, sheep breeds, size of pastures, and worm-control practices: treatment times and frequency, products, and dosages of anthelmintic drugs. The surveyed farms were situated in western (12.7 %), central (40.8 %), and eastern (46.5 %) Lithuania. Because of the appropriate farm management and treatment strategy twenty five sheep farms from the questionnaire survey were selected for the further research.

Selection of sheep flocks

A total of 25 sheep farms, mainly in central and southern Lithuania, were visited between April 2014 and November 2014. Eighteen of these farms were included in the study and seven were excluded due to faecal egg counts (FECs) <140 eggs per gram (EPG). Flocks were selected randomly and identified by GIN FECs. Faecal samples were collected from the rectums of 15 randomly selected animals in each flock and analysed using a modified McMaster technique, with a minimum sensitivity of 20 EPG (Roepstorff & Nansen, 1998). The selected flocks had been regularly treated with anthelmintics (Jackson *et al*., 2012). All flocks had grazing animals at the time of the study. The last anthelmintic treatment had been given at least eight weeks before the beginning of the study. The selected flocks consisted of approximately 40 – 500 animals.

FECRT

Sheep older than 18 months in each flock were divided into two groups of 15 animals, marked with different coloured sprays, and treated with two different anthelmintics. The number of animals in each group was based on the recommendation that 10 animals per group were sufficient to detect differences in FECs between groups (Coles *et al*., 1992). We chose 15 animals per group to guard against any losses of faecal samples (Falzon *et al*., 2013).

We selected the BZ and ML anthelmintic classes for evaluation in this survey, based on the information obtained from the questionnaire. One group in each flock was treated with the BZ fenbendazole (FBZ) at 7.5 mg/kg body weight (Panacur® granules, Intervet International B.V., Boxmeer, Netherlands), and the other group was treated with IVM at 0.2 mg/kg body weight (Biomectin 1 %, Vetoquinol Biowet Sp.zo.o., Gorzow Wlkp., Poland). The doses were based on the heaviest animal in each flock. FBZ was administrated orally over the back of the tongue, and IVM was administrated subcutaneously behind the scapula. Five of the 18 flocks received only one anthelmintic (IVM on three farms and FBZ on two farms), because of the difficulties of management. Individual faecal samples were collected on the day of treatment (T1). Animals with <140 EPG on day zero (T1) were removed from the trial. Eggs of *Nematodirus* spp. were not included in the counts. Individual post-treatment faecal samples (T2) were collected after 14 days from both groups in each flock treated with both anthelmintics, after 10 days from the flocks treated with only FBZ, and after 14 days from the flocks treated with only IVM. The FEC reduction (FECR) was calculated as:

$$FECR\ (\%) = 100 \times (1 - (T2/T1))$$

where T2 is the arithmetic mean FEC post-treatment and T1 is the arithmetic mean FEC pre-treatment.

Larval cultures

Post-treatment larval cultures were prepared from pooled faecal samples for the flocks with FECRT efficacies <100 %. The pooled samples were composed of the faeces collected from each animal of the group. Ten grammes of faeces were mixed with 4 g of vermiculite and incubated for 7 d at 27 °C (water was added to maintain an adequate moisture level). Third-stage larvae (L3) were then recovered from the coprocultures by a Baermann technique (Coles *et al*., 1992). The L3 were morphologically differentiated and identified according to MAFF (1986) and Van Wyk *et al*. (2004). The first 100 L_3, or all L_3 when <100 developed, were identified.

Statistical analysis

The FECR and the lower limit for a 95 % confidence interval were calculated following the WAAVP recommendations (Coles *et al*., 1992). Flocks with FECRs <95 % and lower limits <90 % were considered as harbouring GINs resistant to an anthelmintic. If only one of these conditions was met, resistance was suspected. Descriptive statistics were calculated using Microsoft® Excel 2007 and IBM SPSS Statistics (Version 21.0).

This study complied with Lithuanian animal welfare regulations

(No. B1-866, 2012; No. XI-2271, 2012) and was approved by the Lithuanian Committee of Veterinary Medicine and Zootechnics Sciences (Protocol No.07/2010).

Results

Farm questionnaire

All farms were mainly specialized for meat production and practiced grazing, and 19.7 % of the farms had an ecological status. Rotational grazing was used on 78.3 % of the farms, and 21.7 % of respondents kept sheep on the same pasture with a shelter during the grazing period. Pastures had a mean area of 19.53 ha (1-120 ha). The average number of sheep per farm was 149.9 (1-1700). Lithuanian black-headed sheep was the dominant breed (46.5 %). Other breeds were Romanov (15.0 %), German black-headed (13.3 %), Suffolk (8.3 %), and Berichon du Cher (5.0 %). The remaining 11.9 % consisted of breeds such as Merinofleischschaf, Ile de France, Texel, and Lacaune and crossbreeds. Sheep were usually pastured from March/April to October/November, and all sheep were housed during the winter.

An estimated 71.8 % of sheep farmers used anthelmintics against GINs (Table 1), but 9.5 % of farmers declared that they treated their sheep only with the appearance of clinical symptoms such as diarrhoea, apathy and/or weight loss. The most commonly used classes of anthelmintics were MLs (68.6 %), and BZs (27.5 %).

Table 1. Worm-control practices on the sheep farms

Worm-control factor	Number (%)
Anthelmintic classes used on sheep farms	
Macrocyclic lactones	29 (56.9)
Benzimidazoles	8 (15.7)
Imidazothiazoles	2 (3.9)
Macrocyclic lactones and benzimidazoles	12 (23.5)
Treatment frequency (yearlings/ewes)	
None	20 (28.2)
Once	7 (9.8)
One to two times	9 (12.7)
Twice	32 (45.1)
Two to three times	1 (1.4)
Three to four times	2 (2.8)
Treatment frequency (lambs)	
None	20 (28.2)
Once	16 (22.5)
One to two times	3 (4.2)
Twice	30 (42.3)
Two to three times	2 (2.8)

From the BZs group 68.7 % albendazole and 31.3 % FBZ were used. Levamisole was used very sporadically (3.9 %) (Table 1).

Spring before turn out and autumn before turn in were the most common times to treat ewes. Yearlings and adults were usually treated together, with a mean annual drenching rate of 1.39. Most of the respondents declared that they treated lambs at the same time as the yearlings and adults to save time, with a mean number of treatments of 1.24 (Table 1). Of the respondents that used anthelmintics, 62.7 % declared that they treated their animals twice every year. All respondents that used anthelmintics once per year treated their ewes in spring. A few farms added a treatment in summer (7.1 %). Anthelmintics were rotated on 4.8 % of the farms. Only one respondent reported four treatments per year. Annual treatments were usually performed without any parasitological analyses, and 11.9 % of the respondents reported only a single coprological analysis during the entire period when sheep were kept. Animal weights were visually appraised on 92.9 % of the farms, and only 7.1 % of farmers weighed their animals. Veterinarians treated the animals on 54.8 % of the farms, and owners or farm workers treated the animals on 45.2 % of the farms. Problems with sheep GINs were declared by 39.2 % of the respondents.

Evaluation of the FECRTs

The arithmetic mean FECs, percentages of the FECRs and 95 % confidence intervals are presented in Table 2. The FECRs indicated the presence of FBZ resistance on three of the 15 farms where FBZ was used (FECRs ranged from 44.9 to 85 %), and FBZ resistance was suspected on one farm (94.3 %, CI 93-99). Resistance to IVM was present on two of the 16 farms that used IVM (FECRs of 74.9 and 77.2 %). On one farm (Farm No.7) resistance was detected to both classes of anthelmintics. Mean pre-treatment EPG counts varied from 216 to 3114.

The main species of resistant GIN identified after treatment were *Teladorsagia* spp. On all 6positive farms, with distribution varying from 42 to 100 %. *Trichostrongylus* spp. was found on five farms, with distribution varying from 4 to 100 %, and 6-56 % of the GINs on seven farms were *Cooperia* spp. *Chabertia ovina* was found on two farms (4-18 %), and *Haemonchus contortus* was found on only one farm (10 %) (Table 3).

Discussion

This study is the first to investigate the presence of AR in Lithuania. The number of sheep raised is increasing each year, and AR problems have begun to appear. This study demonstrated that AR to GINs in sheep occurs in Lithuania but to a lower extent than in Australia, New Zealand, South Africa, and other countries. AR in sheep varies widely in various countries of Europe. AR to BZs was found on 83 % of the sheep farms examined in western France (Chartier *et al*., 1998), 11.0 % in Norway (Domke *et al*., 2012), 13.6 % of AR to BZs and 27.3 % to IVM in Spain (Martinez-Valladares *et al*., 2013). In our study, the FECRTs indicated that AR was present in

Table 2. Mean eggs per gram of faeces (EPG), faecal egg count reduction percentages (FECR%) and 95% confidence intervals (CI) on 18 sheep farms in Lithuania treated with fenbendazole (7,5 mg/kg) and ivermectin (0,2 mg/kg)

Farm no.	Anthelmintic class (drug)	EPG (range) pre – treatment	EPG (range) post – treatment	FECR% CI
1	FBZ	596 (160 – 1340)	34 (0 – 140)	94.3 (93 – 99)
	IVM	300 (140 – 600)	10 (0 – 100)	96.7 (94 – 100)
2	FBZ	1346 (160 – 3540)	26 (0 – 100)	98.1 (97 – 100)
	IVM	446 (160 – 1040)	0	100
3	FBZ	378 (140 – 1240)	0	100
	IVM	415 (140 – 1080)	0	100
4	FBZ	544 (140 – 2120)	82 (0 – 220)	85 (73 – 98)
	IVM	332 (140 – 1000)	2 (0 – 20)	99.4 (99 – 100)
5	FBZ	3114 (140 – 11720)	120 (0 – 940)	96.2 (95 – 100)
	IVM	2843 (140 – 19760)	0	100
6	FBZ	264 (140 – 640)	0	100
	IVM	267 (140 – 420)	0	100
7	FBZ	278 (140 – 620)	68 (0 – 140)	75.6 (66 – 85)
	IVM	428 (160 – 1080)	98 (0 – 520)	77.2 (68 – 89)
8	FBZ	n.d.	n.d.	n.d.
	IVM	736 (140 – 3120)	0	100
9	FBZ	1366 (160 – 6680)	0	100
	IVM	788 (140 – 2660)	0	100
10	FBZ	352 (140 – 1060)	6 (0 – 40)	98.3 (96 – 100)
	IVM	216 (140 – 400)	4 (0 – 40)	98.2 (98 – 100)
11	FBZ	287 (140 – 620)	0	100
	IVM	n.d.	n.d.	n.d.
12	FBZ	n.d.	n.d.	n.d.
	IVM	388 (140 – 680)	0	100
13	FBZ	783 (140 – 2960)	432 (60 – 1360)	44.9 (31 – 56)
	IVM	n.d.	n.d.	n.d.
14	FBZ	414 (140 – 1100)	0	100
	IVM	246 (140 – 560)	0	100
15	FBZ	2724 (140 – 15120)	0	100
	IVM	1080 (160 – 3600)	272 (0 – 2140)	74.9 (65 – 84)
16	FBZ	223 (140 – 680)	0	100
	IVM	1044 (140 – 6840)	0	100
17	FBZ	n.d.	n.d.	n.d.
	IVM	238 (140 – 760)	0	100
18	FBZ	725 (140 – 2680)	0	100
	IVM	868 (140 – 3240)	0	100

Telad.: Teladorsagia; Trich.: Trichostrongylus; Chab.: Chabertia; Haem.: Haemonchus; Coop.: Cooperia
n.d.: not done

Table 3. Third-stage larvae (L3) identified in post-treatment coprocultures on 6 sheep farms with AR in Lithuania treated with fenbendazole (7,5 mg/kg) and ivermectin (0,2 mg/kg)

Farm no.	Anthelmintic class (drug)	Larval identification (% L_3) post-treatment				
		Telad.	Trich.	Chab.	Haem.	Coop.
1	FBZ	88	-	-	-	12
	IVM	-	100	-	-	-
4	FBZ	58	42	-	-	-
	IVM	100	-	-	-	-
5	FBZ	40	-	-	10	50
	IVM	-	-	-	-	-
7	FBZ	44	-	-	-	56
	IVM	42	4	4	-	50
13	FBZ	52	28	-	-	20
	IVM	n.d.	n.d.	n.d.	n.d.	n.d.
15	FBZ	-	-	-	-	-
	IVM	54	22	18	-	6

Telad.: Teladorsagia; Trich.: Trichostrongylus; Chab.: Chabertia; Haem.: Haemonchus; Coop.: Cooperia
n.d.: not done

27.8 % of the flocks, independent of the anthelmintic used. Sheep GINs were resistant to IVM on 12.5 % of the farms and to FBZ on 20 % of the farms, with suspected resistance on one farm (6.7 %). One farm (Farm No. 13) in our study had a very high level of AR to BZs. The owner had been treating the sheep 2-3 times a year for nearly 10 years with the same group of anthelmintics. The lack of rotation of anthelmintics clearly led to the development of AR. In our study, on one farm (6.7 %) multi-drug resistance was detected. The FECRT is the most common method to diagnose AR but only detects resistance when at least 25 % of the population is resistant (Martin *et al*., 1989; Martinez-Valladares *et al*., 2013).

The isolation of L_3 from post-treatment coprocultures from flocks resistant to BZs and MLs indicated that the most prevalent parasites were *Teladorsagia* and *Trichostrongylus*, as also reported by Bartley *et al*. (2003), Cernanska *et al*. (2006), and Martinez-Valladares *et al*. (2013). *Cooperia* and *Chabertia*, and *Haemonchus* on one farm, were also found. *Haemonchus contortus* is one of the most pathogenic GIN species in sheep, and AR in *H. contortus* is widespread throughout the world in sheep and goats (Cernanska *et al*., 2006). Our study, however, showed that this parasite is not very common in Lithuania.

The survey showed that a higher number of sheep farmers in Lithuania are using anthelmintics. Knowledge of AR, the prevalence of GINs, and proper usage of anthelmintics, however, is lacking. IVM is the most popular anthelmintic in Lithuania (68.6 %). FBZ is the only registered BZ in Lithuania, but farmers often choose albendazole from other countries because of its low cost and easy administration. Levamisole is used very rarely in Lithuania, as in other European countries (Cernanska *et al*., 2008).

The most common feeding system with sheep is semi-intensive grazing on natural pastures, and housing and additional feeding are provided during winter. The pasture season in Lithuania lasts ca. 200 days. Our study indicated that the majority of farms keep Lithuanian black-headed sheep (46.5 %). The mean annual drenching rates were 1.24 and 1.39 for lambs and yearlings/ewes, respectively. Lambs and ewes were drenched 3.2 and 2.7 times per year in Scotland (Bartley *et al*., 2003), 1.76 and 1.70 times per year in the Slovak Republic (Cernanska *et al*., 2008), and 1.9 and 2.3 times per year in Denmark (Maingi *et al*., 1996), respectively. The mean frequency of drenching in France was 5.2 times per year (Chartier *et al*., 1998). Sheep farmers in Lithuania drench their sheep less often relative to these countries.

Visual appraisal of animal weight was based mostly on an average weight. Underestimation of real weights can lead to under-dosing, which can contribute to the development of AR (Chartier *et al*., 1998). A better option would be to dose based on the weight of the heaviest animal (Waller, 1987). Only 7.1 % of the farmers in our survey weighed their sheep before drenching. Many of the farmers also did not rotate anthelmintics for many years but always used the same class of anthelmintics (56.9 % MLs and 15.7 % BZs), which can also contribute to the development of AR. The results from this study confirmed the presence of AR to GIN infection on sheep farms in Lithuania. Future studies should assess AR to GIN infection using *in vitro* methods.

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