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The effect of risk factors of sheep flock management practices on the development of anthelmintic resistance in the Czech Republic

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ABSTRACT

The in vivo faecal egg count reduction test was used for the evaluation of the benzimidazole and macrocylic lactone drug efficacies against gastrointestinal nematodes in 10 flocks of sheep. The same samples were tested concurrently in vitro for benzimidazole resistance using the egg hatch test, and for resistance to ivermectins using the microagar larval development test. The conformity of obtained results between in vivo and in vitro tests was recorded. When the selected methods were applied, anthelmintic resistance to benzimidazoles was detected at four (40%) farms while resistance to ivermectins was evident at one (10%) farm. At one farm (10%) ivermectin resistance was suspected. Moxidectin was effective at all surveyed farms. *Teladorsagia* was recognized as the only benzimidazole resistant genus in post-treatment coprocultures, whereas *Haemonchus* larvae were resistant to ivermectins. This represents the first recorded occurrence of ivermectin resistance in gastrointestinal nematodes of sheep in the Czech Republic. A linear mixed-effects model demonstrated that the majority of evaluated management practices have a significant effect on resistance to benzimidazoles. While application of preventive practices like quarantine and smart drenching maintains low levels of anthelmintic resistance, others like the dose-and-move strategy, administration of the same family of drugs over extended periods of time, and number of treatments per year are responsible for the increase of resistance to anthelmintics at evaluated farms. Only targeted selective treatments approaches had no effect on resistance status. This study indicates the importance of farm management practices in anthelmintic resistance development.

1. Introduction

Parasitic gastroenteritis represents a major health problem as well as economic losses in the small ruminant industry worldwide. The control of gastrointestinal nematodes (GINs), which are causative agents of parasitic gastroenteritis, is based primarily on anthelmintic drugs and management practices. However, heavy reliance on anthelmintics and their widespread use select GINs able to tolerate a drug dose that is effective against common populations of nematodes. This anthelmintic resistance (AR) has emerged globally in the sheep and goat industry, and there are currently farms in South Africa and Australia where resistance has become such a serious problem that farming is no longer possible (van Wyk et al., 1989; Sangster and Dobson, 2002; Sutherland and Scott, 2010). Until now, GIN resistance to all major families of broad-spectrum anthelmintics has been detected (Wolstenholme et al., 2004; Cezar et al., 2010). Moreover, multiple resistance to several anthelmintic families has been steadily

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increasing, particularly in countries in the Southern hemisphere (van Wyk et al., 1999; Chandrawathani et al., 2004; Sutherland et al., 2008; Fiel et al., 2011; Veríssimo et al., 2012; Chagas et al., 2013). The status quo in Europe is not so untenable; however, cases of AR have been reported from most European countries (Borgsteede et al., 1997; Bartley et al., 2003; Čerňanská et al., 2006; Taylor et al., 2009; Papadopoulos et al., 2012).

It is generally accepted that resistant genes spread through natural nematode populations, and AR evolves when selection pressure on these nematodes is high (Sangster and Dobson, 2002; Coles, 2005; Sutherland and Scott, 2010). AR evolves rapidly (Wolstenholme et al., 2004), and resistant genes persist in nematode populations for many generations (Palcy et al., 2010). The reversion of these resistant nematodes to susceptibility status is theoretically possible; however, there have been no reports of full reversion in the field to date. Therefore, preventive strategies that slow the development of resistance should be integrated into each breeding management. These strategies include quarantine treatment of incoming animals with two broad spectrum drugs from different anthelmintic families, turning out treated animals to pasture lightly contaminated with nematode eggs and larvae (Fleming et al., 2006; Abbott et al., 2012) and using smart drenching approach to treat only animals that will most benefit from this medicate, so-called targeted selective treatments (Jackson et al., 2009; Kenyon et al., 2009). Moreover, environmental measures including pasture management and weather conditions at a specific farm should also be taken into consideration (Silvestre et al., 2002; Fleming et al., 2006).

Our objective was to evaluate the efficacies of three anthelmintic drugs at selected Czech sheep farms that implement different breeding management and treatment practices, and subsequently to assess the effect of selected risk factors on AR development at these farms.

2. Materials and methods

Altogether 16 sheep farms from different parts of the Czech Republic were visited, and information on farm management strategies practised on these farms was obtained. Of these 16 farms, only ten fulfilled selection criteria, after which their owners agreed to participate in the 2012 study. The major farm management practises evaluated in this study were as follows: (1) treatment of all incoming animals (quarantine); (2) smart drenching approach, which involved weighing animals prior to treatment, correct drug application according to the manufacturer's recommendations, withholding food 24 h prior to drenching and repeated drenching 12 h after the first dose of short acting drugs; (3) drenching only animals that need treatment (targeted selective treatment); (4) using the same anthelmintic drug family in consecutive years; (5) treatment frequency per year; (6) dosing animals with anthelmintics before placing them on the field – the so-called dose and move strategy. Only farmers who follow all recommendations for the smart drenching approach listed above were evaluated as "smart drenching farmers". Faecal consistency was used by farmers as a pathophysiological indicator for the TST approach (see Table 1).

Before initiation of the study, animals on selected farms were examined using the modified Concentration McMaster technique (Roepstorff and Nansen, 1998) with a detection limit of 20 eggs per gram (EPG). Only animals with an EPG of ≥150 were included in the experiment. Sheep from the evaluated farms did not receive any anthelmintic treatment for at least eight weeks prior to the initiation of the study; our study was the first to use moxidectin at these farms. Thirty animals (4–6 months of age) were randomly selected from each farm and divided into three groups of

Table 1
Farm management practises evaluated in relation to anthelmintic resistance.

Farm info	Farm 01	Farm 02	Farm 03	Farm 04	Farm 05	Farm 06	Farm 07	Farm 08	Farm 09	Farm 10
Management system	Ecological	Conventional	Conventional	Conventional	Ecological	Conventional	Conventional	Conventional	Conventional	Conventional
Quarantine new animals	No [a]	Yes	Yes	No	Yes	Yes	No	No	No	Yes
Smart drenching approach	Yes	No	Yes	Partly	Yes	No	Yes	Yes	No	Yes
Targeted Selective Treatments	Yes	No	No	No	Yes	No	Yes	No	Yes	No
Using same drug class in years	(BZs) 1	(BZs) 5	(AVMs) 4	(BZs/AVMs) 4	(BZs) 3	(BZs) 5	(BZs) 3	(BZs) 3	(BZs) 4	(AVMs) 1
Treatment frequency per year	2	4	3	4	2	4	3	4	6	3
Dose-and-move strategy	No	Yes	No	Yes	No	No	No	Yes	Yes	No

[a] A closed flock turnover, benzimidazole (BZ), avermectin (AVM) and moxidectin (MOX).

equal numbers. On day 0, faecal samples from each animal group were obtained and coprologically examined using the Concentration McMaster technique. Each animal was weighed in order to enable correct dosing of anthelmintic drugs and identified by ear-tag numbers.

Benzimidazole (BZ) drug family, a fenbendazole (FBZ), was always used at the recommended dose of 5 mg/kg body weight (Panacur 2.5% susp., Intervet International B.V., The Netherlands) to treat the first group in the flock. An avermectin subfamily – ivermectin (IVM), was applied to the second group of animals (Ecomectin 1% inj., ECO Animal Health Ltd., UK) at 0.2 mg/kg body weight, and the third group was treated with milbemycins–moxidectin (MOX) (Cydectin 0.1% susp., Fort Dodge, UK) at a dose of 0.2 mg/kg body weight. The whole process of AR detection, unless otherwise indicated, was carried out according to the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Coles et al., 1992, 2006). Faecal samples were collected ten days post-treatment for BZs and 14 days post-treatment for IVM and MOX.

The faecal egg count reduction test (FECRT) was used as a gold standard for the evaluation of resistance to BZ, IVM and MOX anthelmintics. Individually based FECR was calculated as follows: $\text{iFECR} = (1/n)\sum(100 \times (1 - [T_{i2}/T_{i1}]))$, where T_{i2} is post-treatment and T_{i1} is pre-treatment EPG in host i from a total n of hosts (Cabaret and Berrag, 2004) and 95% confidence intervals (C.I.) were expressed. Resistance to a specific drug family was considered present if the result of FECR was less than 95%, and the lower C.I. was less than 90%; only one of these two criteria needed to be met in order for resistance to be suspected. Faecal post-treatment cultures were kept in a incubator at 27 °C in moist conditions for one week. *Nematodirus* spp. eggs require special conditions to hatch; therefore, a part of faecal samples was incubated according to van Dijk and Morgan (2007, 2009). The obtained infective larvae (L_3) were identified morphologically to the genus level according to van Wyk et al. (2004).

The egg hatch test (EHT) for in vitro detection of BZ resistance was used. This test was performed as recommended by WAAVP (Coles et al., 1992, 2006; von Samson-Himmelstjerna et al., 2009). A stock solution of thiabendazole (T8904, Sigma–Aldrich) was dissolved in dimethylsulfoxide (DMSO), and the following final thiabendazole concentrations were used: 0.05, 0.1, 0.2, 0.3 and 0.5 μg/ml. An egg suspension (100 eggs/ml) was applied to each well of the 24-well plates, which contained the specific thiabendazole concentration. Each sample was tested in duplicate, and two control wells without anthelmintic drug were included. Sealed plates were incubated for 48 h at 25 °C. The incubation was terminated by adding 10 μl of Gram's iodine to each well. Finally, the number of eggs and larvae in each well was determined, and the concentration of thiabendazole required to kill 50% of the eggs (EC_{50}) was calculated. A flock was classified as resistant to BZ drugs if the EC_{50} was over 0.1 μg/ml.

The IVM resistance was evaluated in vitro using a modified version of the microagar larval development test (MALDT) described by Dolinská et al. (2012). A stock solution of ivermectin aglycone (Tebu-bio) was diluted with DMSO and was placed into the wells of a micro-plate together with 150 μl of 2% Bacto agar to produce ten final drug concentrations (0.05–100 ng/ml). After solidification of the agar, 100 eggs in an Amphotericin B solution were mixed with 10 μl of yeast/Earl's extract enriched with *Escherichia coli* suspension and added to the wells. Each sample was tested in duplicate, and two control wells without the drug were included. The sealed plates were then incubated at 25 °C for 7 days, after which a drop of Gram's iodine was added to each well to kill the larvae. The number of eggs and larvae was estimated, and the concentrations of ivermectin aglycone that inhibited development of 50% (LC_{50}) and 99% (LC_{99}) of larvae were calculated. After cultivation, the L_3 were identified to the genus level according to van Wyk et al. (2004). *Haemonchus* spp. was classified as resistant to IVM drugs if the LC_{50} was >5.4 ng/ml and the LC_{99} was >10.9 ng/ml, whereas the discriminating dose for IVM resistant *Teladorsagia* and *Trichostrongylus* species was four times higher – LC_{50} was 21.6 ng/ml and LC_{99} was 86.4 ng/ml.

The influence of particular major preventive practises on AR status was evaluated by a linear mixed-effects model. Due to the limited data set, the individual results of the FECRT from all tested animals were utilized. The farm was, therefore, used as a superior factor with a random effect in linear mixed-effects models. The following factors with fixed effects were utilized: (1) the quarantining of new animals; (2) the smart drenching approach; (3) the use of the same family of drugs over long periods of time; (4) number of treatments per year; (5) targeted selective treatment and (6) the dose-and-move strategy. The starting maximal model contained all these factors. Interactions of factors were not included in the starting model because their testing was due to character and relations among factors meaningless. During the following model simplification, models using ANOVA and the maximum likelihood method (Crawley, 2007) were compared. Variables included in the minimal adequate model were recognized as factors with influence on AR. These factors were then visualized, and their effects on AR risk were evaluated.

Only BZ influence was tested due to fact that individual variability of IVM resistance was low and observed only at one of the farms (farm 04); furthermore, MOX was effective in all tested sheep flocks. Statistical significance was established using $\alpha = 0.05$. Each minimal adequate model was evaluated using standard statistical diagnostics, i.e., by residuals and standardized residuals versus fitted and predicted values (Crawley, 2007; Pekár and Brabec, 2009). All tests were computed using R statistical software, version 2.15.1 (R Development Core Team, 2012).

3. Results

Treatment efficacies of BZ, IVM and MOX used in this study in a single active formulation are presented in Table 2. The average pre-treatment FEC detected in sheep included in the study varied from an EPG value of 121.4 to 845.7. Infective larvae detected in the pre- and post-treatment coprocultures are listed according to their intensity in Table 3. Mixed infections were observed in all pre-treatment samples. The most prevalent genera were *Trichostrongylus* and *Teladorsagia*, followed by *Haemonchus*, *Nematodirus*, *Chabertia* and *Oesophagostomum*. *Cooperia* was detected only at a low frequency.

BZ resistance was detected at four evaluated farms (02, 04, 08 and 09) using in vivo test (FECR 91.3%, 91.4%, 80.5% and 68.9%). An FECR below 95%, which indicated IVM resistance, was observed at farm 04. At farm 03, IVM resistance was merely suspected (FECR 94.9%, lower C.I. 88.6%). Treatment with MOX was effective at all evaluated farms (FECR 96.6–100%). *Trichostrongylus*, *Teladorsagia*, *Haemonchus*, *Nematodirus* and *Chabertia* larvae were identified in post-treatment coprocultures. *Teladorsagia* was evaluated as the only one genus resistant to BZ drugs. On farm 04, resistance to IVM drugs in *Haemonchus* was determined.

Using the EHT, BZ resistance was detected at four farms (EC_{50} 0.136, 0.291, 0.148 and 0.412 μg/ml). All results of this in vivo BZ resistance detection test coincided with those of the FECRT. The MALDT (LC_{50} 5.9 and LC_{99} 64.3 ng/ml) as well as the FECRT (78.0%) revealed resistance to IVM drugs at farm 04. However, the in vitro method for the detection of resistance to IVM has yet to be standardized, nor have the results been verified for all GIN genera, and any obtained results should be interpreted with caution.

When certain major preventive practises are tested, there is indication that only targeted selective treatment has no influence on BZ resistance (GLMM: $\chi^2 = 0.154$, $df = 1$, $P = 0.695$). The risk of resistance decreased on farms where quarantine treatment ($\chi^2 = 8.303$, $df = 1$, $P < 0.01$) and smart drenching approach ($\chi^2 = 6.428$, $df = 1$, $P < 0.001$) were applied. Conversely, the risk of resistance increased on farms where a dose-and-move strategy ($\chi^2 = 6.851$, $df = 1$, $P < 0.05$) was practised. There was also an increase in the risk of resistance with prolonged using of the same anthelmintic family ($\chi^2 = 12.667$, $df = 1$, $P < 0.001$) and with increased treatment frequency per year ($\chi^2 = 6.335$, $df = 1$, $P < 0.05$).

Table 2
Results of in vivo and in vitro tests for the detection of anthelmintic resistance in sheep flocks treated with three anthelmintic drug families.

Farm	Anthelmitic family	Pre-treatment FEC	Post-treatment FEC	FECR (CI)	Resistance status	EHT	MALDT	
		FEC ± S.E.M.	FEC ± S.E.M.			EC_{50}	LC_{50}	LC_{99}
01	BZ	367.9 ± 158.2	10.7 ± 6.2	96.2 (94.9–99.1)	S	0.047		
01	AVM	336.6 ± 140.9	0 ± 0	100	S		16.3	77.1
01	MOX	475.2 ± 211.6	0 ± 0	100	S			
02	BZ	445.7 ± 127.5	32 ± 25.6	91.3 (86.5–97.4)	R	0.136		
02	AVM	190.0 ± 75.1	2.1 ± 1.3	98.0 (96.0–99.5)	S		14.1	53.5
02	MOX	431.5 ± 198.3	9.4 ± 7.6	97.3 (91.6–98.2)	S			
03	BZ	293.3 ± 130	0 ± 0	100	S	0.025		
03	AVM	241.3 ± 115.7	11.2 ± 5.8	94.9 (88.6–98.9)	SR		20.7	85.6
03	MOX	320.0 ± 189.3	0 ± 0	100	S			
04	BZ	335.4 ± 99.1	29 ± 26.1	91.4 (82.3–97.1)	R	0.291		
04	AVM	348.9 ± 203.4	71.2 ± 39.4	78.0 (63.6–93.8)	R		5.9	64.3
04	MOX	422.8 ± 184.2	17 ± 6.6	98.9 (92.6–99.9)	S			
05	BZ	168.2 ± 73.6	0 ± 0	100	S	0.049		
05	AVM	246.4 ± 130.7	8.9 ± 6.1	97.1 (91.8–97.9)	S		17.2	66.0
05	MOX	121.4 ± 87	4.1 ± 1.5	96.4 (92.3–98.5)	S			
06	BZ	225.3 ± 134.8	6.9 ± 2	97.5 (93.1–99.4)	S	0.074		
06	AVM	161.3 ± 76.4	0 ± 0	100	S		13.8	74.9
06	MOX	266.4 ± 103.5	0 ± 0	100	S			
07	BZ	845.7 ± 127.5	8.6 ± 3.9	98.2 (93.6–99.5)	S	0.062		
07	AVM	735.4 ± 112.7	5.9 ± 3.4	96.9 (95.3–100)	S		15.3	53.3
07	MOX	780.2 ± 205.1	0 ± 0	100	S			
08	BZ	348.6 ± 87.9	65.9 ± 28.3	80.5 (76.8–92.9)	R	0.148		
08	AVM	337.3 ± 106.2	0 ± 0	100	S		14.9	46.1
08	MOX	392.5 ± 111.0	0 ± 0	100	S			
09	BZ	466.4 ± 193.4	143.8 ± 72	68.9 (48.6–85.3)	R	0.412		
09	AVM	558.9 ± 205.8	0 ± 0	100	S		11.7	37.4
09	MOX	420.0 ± 164.1	1.8 ± 0.2	98.9 (97.2–100)	S			
10	BZ	407.3 ± 173.9	11.1 ± 6.0	98.2 (94.3–98.5)	S	0.059		
10	AVM	488.5 ± 147.6	3.2 ± 1.4	99.0 (96.3–99.4)	S		13.4	58.0
10	MOX	539.0 ± 215.2	0 ± 0	100	S			

Benzimidazole (BZ), avermectin (AVM) and moxidectin (MOX), pre- and post-treatment mean faecal egg counts, S.E.M. – standard error about the mean, CI – confidence interval, resistance status – susceptible (S), suspected resistance (SR), resistance (R), Egg hatch test – thiabendazole concentration in μg/ml which prevent hatching of 50% eggs (EC_{50}), microagar larval development test – ivermectin concentration in ng/ml which inhibit development of 50% (LC_{50}) and 99% (LC_{99}) infective larvae.

4. Discussion

In the past decade, reports of AR from all areas of sheep production have increased. The first occurrence of BZ resistance in GINs in the Czech Republic was recorded by Chroust (1998), who detected lowered FECR values (61.5% and 84.1%) in selected sheep flocks; nevertheless, information regarding AR prevalence is lacking. The same author (Chroust, 2000) revealed fenbendazole resistance in *Teladorsagia* spp. and *Trichostrongylus* spp. nematodes at two (FECR 83.7% and 85.4%) of three evaluated sheep farms in the Czech Republic. A recently published survey (Vernerová et al., 2009) detected BZ resistance at two of four Czech sheep farms. Our study detected, BZ resistance in four of ten evaluated sheep flocks using the FECRT and EHT. On one farm, the ability of GINs to tolerate the recommended dose of IVM was revealed applying in vivo as well as in vitro tests; this was the first time IVM resistance was recorded in the Czech Republic. On another farm the IVM resistance was merely suspected. Neither these results nor the results of the above-mentioned studies can correctly describe the AR situation in the Czech Republic. For these purpose the evaluation of more farms is necessary, and such studies for this region are highly welcomed. Nevertheless, assessing the factors influencing resistance in GINs at selected farms was the main goal of our study, rather than merely monitoring AR prevalence. The effect of these risk factors is discussed below.

The treatment of all in-coming animals is a key factor in preventing the introduction of resistant nematodes to farm. In our study, AR was detected at three of four farms that do not use quarantine treatment. The absence of quarantine treatment was evaluated as an important risk factor for the establishment of AR. Quarantine treatment with two broad spectrum anthelmintics is widely recommended (Fleming et al., 2006; Abbott et al., 2012). This synergistic effect of applied drugs increases the efficacy of treatment and reduces the probability of survival for resistant nematodes. When no external genetic material (animals) is imported to the farm, a closed flock turnover, practiced at farm 01 can also prevent the introduction of resistant alleles to the flock.

The smart drenching approach employs a spectrum of knowledge regarding host physiology, anthelmintic pharmacokinetics, parasite biology, etc., which maximizes anthelmintic efficacy and concurrently reduces selection pressure for AR (Fleming et al., 2006). This approach is especially important for short acting drugs, which, like BZs, are drenched orally. Not following this preventive measure also had a significant effect on the resistance level in sheep flocks in our study. Sheep on these farms had access to food during the treatment and drugs were administered as

Table 3
Infective larvae of gastrointestinal nematodes recovered from pre- and post-treatment coprocultures in sheep treated with three anthelmintic drug families.

Farm	Anth. family	Coproculture pre-treatment	Resistance status	Coproculture post-treatment
01	BZ	*Tel*, *Hae*, *Tri*, *Nem*, *Coo*	S	*Tel*, *Tri*, *Hae*
01	AVM	*Tel*, *Tri*, *Hae*, *Nem*, *Oe*, *Coo*	S	0
01	MOX	*Tel*, *Tri*, *Hae*, *Nem*, *Oe*, *Coo*	S	0
02	BZ	*Tri*, *Tel*, *Nem*, *Oe*, *Coo*	R	*Tel*
02	AVM	*Tri*, *Tel*, *Nem*, *Oe*, *Cha*	S	*Tel*, *Tri*, *Nem*
02	MOX	*Tri*, *Tel*, *Nem*, *Oe*, *Coo*	S	*Tri*, *Tel*, *Nem*
03	BZ	*Tri*, *Tel*, *Cha*, *Coo*, *Oe*	S	0
03	AVM	*Tel*, *Tri*, *Cha*, *Oe*, *Coo*	SR	*Tel*
03	MOX	*Tri*, *Tel*, *Cha*, *Oe*, *Coo*	S	0
04	BZ	*Tri*, *Tel*, *Hae*, *Cha*	R	*Tel*
04	AVM	*Tri*, *Tel*, *Hae*, *Cha*	R	*Hae*
04	MOX	*Tel*, *Tri*, *Hae*, *Cha*	S	*Tel*, *Hae*
05	BZ	*Tri*, *Tel*, *Oe*, *Cha*, *Nem*, *Coo*	S	0
05	AVM	*Tel*, *Tri*, *Oe*, *Coo*, *Nem*, *Cha*	S	*Tel*
05	MOX	*Tri*, *Tel*, *Oe*, *Cha*, *Nem*, *Coo*	S	*Tel*
06	BZ	*Tri*, *Tel*, *Nem*, *Hae*, *Oe*	S	*Tel*, *Tri*, *Hae*
06	AVM	*Tri*, *Tel*, *Nem*, *Hae*, *Oe*	S	0
06	MOX	*Tri*, *Tel*, *Nem*, *Hae*, *Oe*	S	0
07	BZ	*Tri*, *Tel*, *Oe*, *Cha*, *Coo*	S	*Tel*, *Tri*, *Cha*
07	AVM	*Tri*, *Tel*, *Oe*, *Cha*, *Coo*	S	*Tel*, *Tri*, *Cha*
07	MOX	*Tel*, *Tri*, *Oe*, *Cha*, *Coo*	S	0
08	BZ	*Tri*, *Tel*, *Cha*, *Hae*, *Oe*	R	*Tel*
08	AVM	*Tel*, *Tri*, *Cha*, *Hae*, *Oe*	S	0
08	MOX	*Tri*, *Tel*, *Cha*, *Oe*, *Hae*	S	0
09	BZ	*Tel*, *Tri*, *Hae*, *Nem*	R	*Tel*
09	AVM	*Tel*, *Hae*, *Tri*, *Nem*	S	0
09	MOX	*Tel*, *Hae*, *Tri*, *Nem*	S	*Tel*, *Tri*, *Hae*
10	BZ	*Tri*, *Tel*, *Nem*, *Oe*, *Cha*	S	*Tel*, *Tri*
10	AVM	*Tri*, *Tel*, *Oe*, *Nem*, *Cha*	S	*Tel*, *Tri*
10	MOX	*Tri*, *Tel*, *Nem*, *Oe*, *Cha*	S	0

Benzimidazole (BZ), avermectin (AVM) and moxidectin (MOX) drugs, *Tri* – *Trichostrongylus* sp., *Tel* – *Teladorsagia* sp., *Hae* – *Haemonchus* sp., *Nem* – *Nematodirus* sp., *Cha* – *Chabertia* sp., *Oe* – *Oesophagostomum* sp., *Coo* – *Cooperia* sp.; 0 – no larvae found, resistance status – susceptible (S), suspected resistance (SR), resistance (R).

a single dose. However, withholding food for 24 h before oral administration of drug is recommended. A slow digesta flow prolongs the availability of anthelmintics for a parasite, and the efficacy of the drug is improved (Ali and Hennessy, 1995; Abbott et al., 2012). A repeated dose of short acting drugs 12 h apart also increased drug efficacy (Prichard et al., 1978). If these measures are not followed, GINs are exposed to inadequate drug doses; as a result, they survive the treatment, and can further spread resistant alleles.

Altering of the anthelmintic drug family may slow down the selection pressure on a nematode population (Silvestre et al., 2002). An annual (slow) rotation is preferred (Coles and Roush, 1992; Niciura et al., 2012), i.e., when a single drug family is used for one year, and a different anthelmintic is administered for a second year. However, there is no consensus on the use of drug rotation (van Wyk, 2001), and Fleming et al. (2006) suggest that one drug family should be used until it is no longer effective. Anthelmintic rotation within a grazing season such as practised at farm 04 and where resistance to both of the utilized drug families was detected, is not also recommended (Fleming et al., 2006). Our results confirmed a positive correlation between the length of time a particular anthelmintic drug class is administered and the resistance level observed at a farm.

The annual treatment frequency also has an impact on AR development (Barton, 1983; Martin et al., 1984) because the selection pressure on a nematode population is higher (susceptible nematodes have fewer opportunities to survive) when treatments are more frequent. This corresponds to our results, which indicate that AR was detected at farms where sheep were drenched more frequently.

Drenching all animals in the flock concurrently is still common on Czech farms, and half of the evaluated farms practised this approach. This strategy is associated with AR because increases the high resistance genotype frequency (Niciura et al., 2012). Targeted selective treatment (TST) is considered an alternative and more appropriate approach (Cabaret et al., 2009; Cringoli et al., 2009; Busin et al., 2013; Kenyon and Jackson, 2012). The idea of TST is based on negative binomial distribution of GINs in host populations. This means that most animals in the flock carry few parasites, whereas a few heavily infected animals contribute to the total parasite population (Barger, 1985; Sréter et al., 1994). This latter group should be identified according to suitable indicators (Kenyon et al., 2009) and then treated. The animals unexposed to treatment are a source of nematodes not submitted to selection pressure and, thus, contribute to susceptible nematode population that can dilute AR alleles (Sangster and Dobson, 2002). This refugia concept has been widely accepted recently (van Wyk, 2001; Besier, 2012; Jackson et al., 2009). TST using a selected indicator did not have a statistically significant effect on AR status at evaluated farms in our study. There are several indicators more or less suitable for applying the TST approach (see Kenyon et al., 2009). The majority of farmers participating in our study selected faecal consistency as an indicator

for drenching animals. Although it is generally accepted that nematode parasites are a major cause of diarrhoea in grazing animals, diarrhoea is a symptom of many protozoal or bacterial diseases and disorders. Drenching these diarrhoeic animals with anthelmintics is ineffectual, and moreover, selection pressure for resistance increases. This inappropriately selected indicator could explain the failure of the TST approach at evaluated farms.

Even though it was widely recommended in the past and is still applied by many farmers today, the dose and move strategy, in which sheep are dosed with anthelmintics before placing them on a safe pasture, is currently discouraged. This practice selects for AR because nematodes that survive the treatment have a reproductive advantage over susceptible ones (Abbott et al., 2012). In accordance with the opinions expressed by these authors, a significant effect of this strategy was exhibited in this study; AR was detected only at farms where the dose-and-move strategy was applied.

There are possible solutions for farms where AR was detected: (1) treating all animals in the flock using MOX, which is highly effective at these farms; (2) mowing the grass on the contaminated pasture; (3) not allowing animals to graze on such pastures for at least six months. The last step should be to introduce some new sheep, which are lightly infected with susceptible GINs, to this farm in order to dilute resistant alleles, which carry residual infective larvae.

During this study, the highest proportion of larvae recovered from pre-treatment cultures was a member of the *Trichostrongylus* genus. *Teladorsagia* genus was more prevalent only at ecological farm 01. The differences in species prevalence could be explained due to the immune response related to host age (Ross, 1970; Waller and Thomas, 1981). On conventional farms, four-month-old lambs were included in the experiment, and at this age, a larger adult *Trichostrongylus* population can inhibit a smaller *Teladorsagia* population. Older (6 month) lambs were provided for our study by the owner of the ecological farm; at this age, a *Teladorsagia* population can increase dramatically. Moreover, the highest nematode biodiversity was observed at both ecological farms. This corresponds with the view that ecological farming harbours more diverse helminth fauna due to lower pressure caused by anthelmintic treatment (Cabaret et al., 2002). The results of post-treatment cultures together with in vivo test (data not shown) indicate that *Teladorsagia* was the predominant identified genera, which was found to be resistant to fenbendazole at four farms. Most AR monitoring studies (Bartley et al., 2003; Čerňanská et al., 2006; Domke et al., 2012), including those from the Czech Republic (Chroust, 2000), have shown this nematode as a predominant BZ resistant genus. *Haemonchus* was identified as a genera that is resistant to ivermectin. *Haemonchus contortus* is one of the most pathogenic GI nematodes of small ruminants, and it is a dominant species in tropical and subtropical regions (Jabbar et al., 2006). Resistance of this nematode is not only a serious problem in these regions (Wooster et al., 2001; Chandrawathani et al., 2003; Cezar et al., 2010; Tsotetsi et al., 2013) but also in Europe (Scheuerle et al., 2009), and more recently, in Canada (Falzon et al., 2013).

5. Conclusions

Although further research is needed, the results of this study indicate that there are several risk factors promoting development of resistance to anthelmintics. However, it is necessary to appreciate that AR is a complex phenomenon influenced by a number of factors (host, parasite, drugs, management and environment), and clinical resistance to anthelmintic drugs can evolve as a result of synergy among these factors. All of these effects should be taken into account at a specific farm when the efficacy of anthelmintic treatment is evaluated. It is evident that GINs eradication is almost impossible, and man must learn to live with these parasites. Because strict reliance on anthelmintics alone is no longer sustainable, farmers should apply more epidemiological principles in the control of GINs; such measures can slow the development of AR. Nevertheless, changes in the current global climate are affecting epidemiology patterns of helminth parasites in many regions of the world, so the creation of effective preventive measures is a challenge for the future.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All experiments conducted with animals and with anthelmintic drugs were conducted in compliance with the current laws of the country in which they were performed.

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