Detection of benzimidazole resistance in gastrointestinal nematode parasites of sheep in the Czech Republic

E. Vernerova[1], R. Vondrova[2], H. Kisova[2], V. Svobodova[2], A. Hera[1]

[1]Institute for the State Control of Veterinary Biological and Medicaments, Brno, Czech Republic
[2]University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic

ABSTRACT: During 2004–2007 the efficacy of benzimidazole type drugs was studied on 14 sheep farms in the Czech Republic. The study was based on the evaluation of faecal egg count reduction test (FECRT), egg hatch test (EHT), and larval development test (LDT). The prevalence of benzimidazole resistance on farms ranged from 25% to 98%; however, the infection intensity was typically low to moderate on most of the farms. Based of FECRT, resistance was identified on three farms. Resistance was suspected on one of the farms after repeated examination performed the following year.

Keywords: sheep-nematodes; anthelmintic resistance; faecal egg count reduction test (FECRT); egg hatch test (EHT); larval development test (LDT)

Parasitic diseases represent an important issue for all sheep breeders both in the Czech Republic and in other countries. Sheep are the definitive host of gastrointestinal nematodes of genus *Strongyloides* (family Strongyloididae), *Chabertia,* and *Oesophagostomum* (family Chabertiidae), *Bunostomum* (family Ancylostomatidae), *Trichostrongylus, Haemonchus, Ostertagia, Cooperia* and *Nematodirus* (family Trichostrongylidae), and tapeworms of genus *Moniezia expansa* and *Moniezia benedeni* (family Anoplocephalidae). Parasitic diseases caused by these parasites may pose a problem on sheep farms, particularly in countries where sheep farming is highly developed.

In spite of the fact that gastrointestinal parasitic diseases are asymptomatic in the majority of otherwise healthy animals, there are huge financial losses due to decreased milk yield, wool amount and quality, animal vitality and weight gain, and sometimes higher lambs' mortality (Varady and Praslicka, 1993; Rommel et al., 2000). In order to limit these economic losses breeders administer antiparasitic drugs to their animals on a routine basis. This might contribute to the widely occurring parasite resistance to common anthelmintic drugs. This issue appears to be relevant all over the world: there are studies that confirm the rapid evolution and spread of resistant parasites (Besier and Love, 2003; Kaminsky, 2003; Ancheta et al., 2004). Helminths resistant to commonly used anthelmintics have been identified in the Czech Republic (Chroust et al., 1998; Chroust, 2000). Studies conducted in Slovakia, meanwhile, demonstrated that also in this country there are sheep farms with the occurrence of gastrointestinal nematodes resistant to benzimidazoles drugs (Praslicka et al., 1994). Cernanska et al. (2006) found resistant parasites on three out of 25 investigated sheep farms from different regions of the Slovak Republic.

Supported by the Ministry of Education, Youth and Sports of the Czech Republic (Grant No. MSM 6215712403).

Resistance has been defined as an inherited change in the ability of an individual parasite to survive even if exposed to the antiparasitic drug at the common recommended dose, which has been found to be lethal for unselected parasite populations of the same species (Hunt and Taylor, 1989). As a consequence, the usage of the drugs, as well as their load in target animals and the environment and total cost for animal production, is often increased.

The objective of the study was to evaluate parasite infections on sheep farms in the Czech Republic based on faecal examination. The study was focused on the evaluation of the resistance of gastrointestinal nematodes as recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP), using the faecal egg count reduction test (FECRT), egg hatch test (EHT), and larval development test (LDT).

MATERIAL AND METHOD

From April to May over the years 2004 to 2007, faecal samples from 14 sheep farms were investigated by faecal examination (flotation with saturated sucrose solution with specific gravity of 1.3 g/cm^3). 1 222 samples from 14 sheep farms mostly located in Moravia were examined; two farms were located in eastern Bohemia. The mild climate in those areas is influenced by both oceanic and continental effects. For such weather a high variability in temperature and rainfall is typical.

Each sheep farm included in the study had to have at least 50 adult sheep. For each farm, one or two-year-old sheep of either sex were selected and randomly divided into two groups: treated (T) and untreated – control group (C) with 20 (min. 15) sheep in each group. Fenbendazole (Panacur, Intervet; 5 mg/kg bw, *p.o.*, one dose) or albendazole (Aldifal, Mevak, 5 mg/kg bw, *p.o.*, single dose) were given by a calibrated syringe or a drench gun to the sheep of the group. The dosage was estimated according to data given by the sheep owner on the body weight of the animals. The animals included in the study were not allowed to be treated with anthelmitics at least eight weeks before the beginning of the study. All animals were naturally infected. Animals had to have at least 150 eggs per 1 g of faeces and they were chosen from these groups and at least six (four) sheep had to be included in the calculation from these animals.

Detection of anthelmintic resistance

The *in vivo* faecal egg count reduction test (FECRT) – recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) – was used to detect resistance (Coles et al., 1992). The examination was completed with *in vitro* methods, egg hatch test (EHT), and larval development test (LDT) recommended by Varady and Corba (1998, 1999) for field evaluation of antihelmintic resistance.

Faecal egg count reduction test (FECR). On Day 0 (D0), the beginning of the study, faecal samples were collected directly from the rectum of each ewe of the group, by the Group T together with drug administration. After 10 days (D10), the examination was repeated (Coles et al., 2006). At both collection dates the total number of eggs was counted in every animal in 1 g of its faeces (eggs per gram – EPG). Three grams of faeces were weighed out from each sample and were utilized using the McMaster technique with minimal detection limit 50. The faecal samples were stored at 4–6°C until the next day when their evaluation was started. Out of the mean values calculated on Day 10 the percentage reduction was determined using a formula:

$$\text{Percentage reduction} = 100 \times (1 - Xt/Xc)$$

where:

X = the arithmetic mean EPG

t = the treated group at D10

c = the control group at D10

The interpretation of the FECR test results (Coles et al., 1992) was as follows: resistance could be possible if the percentage reduction in egg count is less than 95% and a lower 95% confidence interval is less than 90%. If at least one of those criterions was accomplished, emerging resistance could be suspected.

Egg hatch test (EHT). This test is recommended by the WAAVP (Coles et al., 1992) for the determination of benzimidazole resistance *in vitro*. Samples were collected from the rectum and transported in sample tubes fully filled with water under anaerobic conditions and stored at approximately 20°C till the next day when their processing began. Samples of nematode eggs were homogenized in tap water and purified repeatedly, isolated in distilled water and finally incubated (200 eggs per 1 cm^3) on a 24 well plate with five different concentrations of thiabendazole (0.05–0.25 μg/ml). Thiabendazole

was dissolved in aqueous HCl. One well of the plate did not contain thiabendazole and served as a control. After 48 h incubation the eggs and larvae were counted and ED_{50} determined. Each sample was tested in duplicate and two negative controls were included. Benzimidazole resistance was confirmed if eggs were hatched at a concentration of above 0.1 μg of thiabendazole per ml.

Larval development test (LDT). This test is based on larval ability to survive and develop in various concentrations of the anthelmintic drug. Samples were prepared in the same way as for EHT. Nematodes eggs were incubated on plates for 24 hours. After this time feeding solutions containing *E. coli* and five different concentrations of thiabendazole were added (0.05–0.25 μg/ml). Thiabendazole was dissolved in aqueous HCl. One well of the plate lacked the anthelmintic drug and served as a control. After six days the test was terminated by adding Lugol's iodine. Consequently the percentage of L1, L2 and L3's at each drug concentration were determined. The Minimal inhibitory concentration (MIC) that inhibited the L3 development was assessed. An MIC of above 0.1 μg thiabendazole per ml indicates benzimidazole resistance.

RESULTS

In total 14 sheep farms were examined for the presence of gastrointestinal parasites. On each of the farms, animals positive for the presence of nematode eggs were identified before the first treatment with the anthelmintic drug. Overall prevalence ranged from 48% to 98% in sheep from four farms that were included in the resistance study (Table 1). Out of these farms, resistance was de-

Table 1. Prevalence of gastrointestinal nematodes assessed by epg in sheep farms selected for testing by: FECRT, EHT, LDT

Code of sheep farm	Years of testing	Total prevalence (%)	GIT nematodes* (%)	*Nematodirus* spp. (%)	*Trichuris* spp. (%)
6	2005	78	71	2	7
6	2006	95	60	0	83
7	2006	48	48	4	2
10	2006	50	50	2	2
6	2007	98	98	4	7
12	2007	93	90	0	28

*except genus *Nematodirus* and *Trichuris*, results of which are presented separately in the next columns

Table 2. The percentage of egg number reduction (FECR%) and lower 95% confidence interval (CI) in sheep from four farms that were included in the anthelmintic resistance survey

Code of sheep farm/year	Control group		Treated group		FECR% CI
	number of sheep	EPG D10	number of sheep	EPG D10	
6/05	11	4 354.55 (350–10 750)	11	1 382.73 (150–5 000)	74.71 48.58–87.57
6/06	6	6 733.3 (50–20 800)	7	178.6 (0–750)	92.54 62.94–98.5
7/06	4	50 (0–150)	8	0	100
10/06	9	416.7 (0–1 500)	9	127.7 (0–650)	74.16 11.06–92.49
6/07	13	2 965.4 (0–14 550)	15	57.7 (0–1 400)	98.27 34.60–99.95
12/07	11	586.3 (100–1 500)	8	281.3 (0–950)	68.75 9.64–89.19

tected on three farms (on one of those three farms resistance was suspected based on repeated evaluations). In Table 2, the percentage of the reduction (FECR) and a lower 95% confidence interval (CI) of the four evaluated farms are shown. Parasitological findings on farms 1–5, 8, 9, 11, 13, and 14 were not in accordance with the criterion for the study, i.e., a minimal egg number of 150/1 g of faeces in at least six (four) sheep in a group. From the percentage reduction (FECR%) and lower 95% confidence interval (CI) it can be concluded that on three farms (6, 10, 12) resistance to GI nematodes was found. On farm 6 where the examination was repeated the next year (2007), according to the lower 95% confidence interval it cannot be excluded that resistance was still present in this farm (Table 2). In 2006, on farm 6 and 10 the egg hatch test (EHT) and larval development test (LDT) did not confirm the resistance. The larvae did not hatch at any of the thiabendazole concentrations of EHT nor did any L3 larvae develop in the LDT.

DISCUSSION

Diagnostic procedures focused on parasites of farm animals including sheep are carried out on statistically significant groups of animals rather than individual animals due to considerations of time and cost. The number of sheep bred in the Czech Republic is relatively low, about 300 000 sheep according to statistical data. Most breeders administer antiparasitic drugs as preventive control in spring (March, April) and after pasture (October, November) which seems sufficient to keep the animals free of clinical signs of gastrointestinal nematode infection. Even if the prevalence ranged from 25 to 98%, infection intensity on the majority of farms was low to moderate (0–28 550 epg), and if present, clinical signs were very mild (latent or asymptomatic; Table 1). In addition, the intensity of nematode infections depends on the season of the year. Optimal conditions for hatching and development of the larvae are 18–26°C and 80–100% humidity (Urquhart et al., 1996). These conditions are usually fulfilled during the spring months and at the beginning of summer as L3 larvae have over wintered in pasture and are activated by light and heat (Jurasek and Dubinsky, 1993). Animals become infected when turned out at pasture at the beginning of the season. A significant increase in parasitic infection may be observed when hypobiotic larvae are released (Urquhart et al., 1996). This is the main reason why the survey was carried out in spring, as later it would have become difficult to obtain enough samples with a sufficient number of eggs (i.e. ≥ 150 epg), even if we had included the young animals aged 1–2 years that are predisposed to parasitic infections.

The *in vivo* faecal egg count reduction test (FECRT) was used in our study (Coles et al., 1992). Based on our results (see Table 2) resistance to benzimidazoles was detected in farm numbers 6, 10 and 12. Resistance was also confirmed by the *in vitro* egg hatch test (EHT) that is thought to be highly sensitive (Martin et al., 1982, 1984; Barton, 1983; Presidente, 1985) but not recommended for use with *Nematodirus* spp., where a standardised egg embryonation test is best (Coles et al., 2006). However, previous studies indicate that anthelmintic resistance in parasitic nematodes may not be identified by the test if the studied population of parasites does not include at least 25% of resistant nematodes genus *Trichostrongylus* and more than 50% of nematodes genus *Ostertagia* (Hunt and Taylor, 1989; Martin et al., 1989). Accordingly, if the number of resistant nematodes is low in the population, the test may fail to detect resistance (Varady et al., 1995; Varady and Corba, 1998). In our study (carried out on 6/06, 10/06) no hatching occurred at any of the thiabendazole concentrations (i.e., development of L3) neither when the egg hatch test (EHT) or larval development test (LDT) were performed. Neither EHT nor LDT indicated benzimidazole resistance. The discrepancy between the results of the *in vivo* and *in vitro* tests could be due to the different substances used for testing. Thiabendazole was used in the EHT and LDT, however, albendazole (farms 6/06, 10/06, 12/06, 13/07, 6/07) or fenbendazole (study 6/05) was used as treatment in the FECRT. It also must be emphasized that thiabendazol, used as a model substance in accordance with the WAAVP's recommended method had ovicidal activity. This is a particular advantage of the *in vitro* EHT, in contrast to the *in vivo* FECRT that focuses on evaluation of adulticide properties (Varady et al., 1995). This could go some way to explaining the discrepancy in the results between the tests. It can be stated that the present status on sheep farms represents the beginning of resistance to gastrointestinal nematodes. Sheep in the Czech Republic are typically treated with benzimidazole drugs. One of the reasons for using this anthelmintic drug class is its high efficacy against the tapeworm genus *Moniezia* that

affects lambs in spring. Since no specific drug based on praziquantel, the drug of choice for combating cestode infections in sheep (Kassai, 1999), is authorised in the Czech Republic, breeders are forced to administer either albendazole or fenbendazole. Antiparasitic drugs must be administered more frequently also because of the limited opportunity of changing pastures and higher concentration of animals on the pastures. These were the conditions on farms 6, 10 and 12, in contrast to the majority of studied farms (where sheep returned to the same pasture every other year).

It can be summarized that the intensity of gastrointenstinal nematode infections and the prevalence of benzimidazole resistance in gastrointestinal nematodes in sheep in studied farm are relatively low. Even if the findings could be viewed as reassuring for Czech sheep breeders, the increasing number of resistant nematodes confirmed on some of the farms in the EU, demonstrates the necessity of following all preventive measures to avoid the potential spread of resistant gastrointestinal nematodes. Our results can be regarded as a warning sign which should stimulate further investigations with a larger number of farms involved.

REFERENCES

Ancheta P.B., Dumilon R.A., Venturina V.M., Cerbito W.A., Dobson R.J., Le Jambre L.F., Villar E.C., Gray G. D. (2004): Efficacy of benzimidazole anthelmintics in goats and sheep in the Philippines using a larval development assay. Veterinary Parasitology, 120, 107–121.

Barton N.J. (1983): Development of anthelmintic resistance in nematodes from sheep in Australia subjected to different treatment frequencies. International Journal of Parasitology, 13, 125–132.

Besier R.B., Love S.C.J. (2003): Anthelmintic resistance in sheep nematodes in Australia: the need for new approaches. Australian Journal of Experimental Agriculture, 43, 1383–1391.

Cernanska D., Varady M., Corba J. (2006): A survey on anthelmintic resistance in nematode parasites of sheep in the Slovak Republic. Veterinary Parasitology, 135, 39–45.

Chroust K. (2000): Occurrence of anthelmintic resistance in strongylid nematodes of sheep and horses in the Czech Republic (in Czech). Veterinarni Medicina, 45, 233–239.

Chroust K., Horak F., Zizlavsky J., Zizlavska S. (1998): The course and control of parasitoses in mixed grazing of sheep and cattle (in Czech). Veterinarni Medicina, 43,153–159.

Coles G.C., Bauer C., Borgsteede F.H.M, Geerts S., Klei T.R., Taylor M.A., Waller P.J. (1992): World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary Parasitology, 44, 35–44.

Coles G.C., Jackson F., Pomroy W.E., Prichard R.K., von Samson-Himmelstjerna G., Silvestre A. (2006): The detection of anthelmintic resistance in nematodes of veterinary importance. Veterinary Parasitology, 136, 167–185.

Hunt K.R., Taylor M.A. (1989): Use of the egg hatch assay on sheep faecal samples for the detection of benzimidazole resistant nematodes. Veterinary Record, 125, 153–154.

Jurasek V., Dubinsky P. et al. (eds.) (1993): Parazitogeografia (in Slovak), 22–24. In: Veterinarna parazitologia. Priroda Press, Bratislava. 382 pp.

Kassai T. (1999): Veterinary Helminthology. 1st ed. Butterworth Heinemann, Oxford. 260 pp.

Kaminsky R. (2003): Drug resistance in nematodes: a paper tiger or real problem? Current Opinion in Infectious Diseases, 16, 559–564.

Martin P.J., Anderson N., Jarret R.G., Brown T.H., Ford G.E. (1982): Effect of a preventive and suppressive control scheme on the development of thiabendazole resistance in *Ostertagia* spp. Australian Veterinary Journal, 58, 185–190.

Martin P.J., Anderson N., Lwin T., Nelson G., Morgan T.E. (1984): The association between frequency of thiabendazole treatment and the development of resistance in field isolates of *Ostertagia* spp. of sheep. International Journal of Parasitology, 14, 177–181.

Martin P.J., Anderson N., Jarret R.G. (1989): Detecting benzimidazole resistance with faecal egg count reduction test and in vitro assays. Australian Veterinary Journal, 66, 236–240.

Praslicka J., Varady M., Corba J. (1994): A survey of anthelmintic resistance in sheep in Slovakia. Veterinary Parasitology, 52, 169–171.

Presidente P.J.A. (1985): Methods for Detection of Nematode Resistance to Anthelmintic. CSIRO Publishing, Melbourne. 13–28.

Rommel M., Eckert J., Kutzer E., Korting W., Schnieder T. (eds.) (2000): Tapeworm attacking (in German), 216–264. In: Veterinarmedizinische Parazitologie. Parey Buchverlag, Berlin. 915 pp.

Urquhart G.M., Armour J., Duncan J.L., Dunn A.M., Jennings F.W. (1996): Veterinary Parasitology. 2nd ed. Blackwell Science Ltd, Oxford. 3–50.

Varady M., Corba J. (1998): Methods of detection of anthelmintic resistance in nematodes of domestic animals. Veterinarni Medicina, 43, 347–354.

Varady M., Corba J. (1999): Comparison of six *in vitro* test in determining benzimidazole and levamisole resistance in *Haemonchus contortus* and *Ostertagia circumcincta* of sheep. Veterinary Parasitology, 80, 239–249.

Varady M., Praslicka J. (1993): Prevalence and species diversity of gastrointestinal nematode in stock of sheep in Slovak (in Slovak). Veterinarstvi, 43, 142–143.

Varady M., Praslicka J., Corba J. (1995): The evaluation of in vivo faecal egg count reduction test and *in vitro* egg hatch test for detection of benzimidazole resistance in sheep nematodes. Helmintologia, 32, 225–228.

Received: 2008–10–14
Accepted after corrections: 2009–10–23

Corresponding Author:

Dr. Eva Vernerova, Institute for the State Control of Veterinary Biological and Medicaments, Hudcova 56a, 621 00 Brno, Czech Republic

Tel. + 420 541 518 231, fax:+ 420 541 212 607, e-mail: vernerova@ukskvbl.cz; ea.vernerova@seznam.cz