ANTHELMINTIC RESISTANCE IN SHEEP FARMS IN LITHUANIA DETECTED BY *IN VITRO* MICRO-AGAR LARVAL DEVELOPMENT TEST

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Abstract. This study was conducted to determine the frequency of anthelmintic resistance (AR) in sheep gastrointestinal nematodes in Lithuania to benzimidazoles and levamisole. The survey was conducted from May 2014 to November 2014. An *in vitro* method Micro-agar larval development test (MALDT) was used for this study. In total, 23 sheep farms were investigated. Seventeen sheep farms were tested for AR to benzimidazoles and six sheep farms were tested for AR to levamisole. The studied sheep flocks consisted of 50-800 animals on each farm. The last anthelmintic treatment had to be carried out at least 10 weeks before the beginning of the study. On each farm, faecal samples from the rectum of 15–20 animals were randomly taken. Resistance to benzimidazoles were found on 12 farms (70.6 %). Resistance to levamisole were found on two farms (33.4 %). This *in vitro* study showed that sheep farms in Lithuania already have problems with AR, especially with resistance to benzimidazoles.

Keywords: anthelmintic resistance, *in vitro*, sheep, benzimidazoles, levamisole

Introduction

Anthelmintic resistance (AR) in gastrointestinal nematodes (GIN) of small ruminants is approaching the dangerous limit. It is a very problematic situation in Australia, New Zealand, Brazil, Uruguay South Africa, where AR exists to one or more anthelmintics (Waller et al., 1996; Cezar et al., 2010; Dolinska et al., 2012). In Europe, AR mainly exists to benzimidazoles and, in sporadic cases, to levamisole (Dolinska et al., 2014). However, AR to macrocyclic lactones has increased over the last decade (Bartley et al., 2003, Traversa et al., 2007; Borgsteede et al., 2007; Papadopoulos, 2008).

It is known, that AR develops because of many reasons, such as no anthelmintic rotation, under-dosing, the intensive use of anthelmintics for the control of helminthic infections (Varady et al. 2011; Cernanska et al., 2008).

Anthelmintic resistance can be detected by *in vivo* and *in vitro* methods. The larval development test is the most widely used an *in vitro* test, based on larval ability to survive and develop in environments of various concentrations of anthelminthics (Varady et al., 2011). Coles et al. (1988) first reported a larval development test that could detect resistance to benzimidazoles and levamisole.

In Lithuania, three broad-spectrum anthelmintic drug classes are most commonly used in sheep: macrocyclic lactones (ivermectin), benzimidazoles (fenbendazole, albendazole) and imidazothiazoles (levamisole). Ivermectin is the most popular anthelmintic in Lithuania. From benzimidazoles only fenbendazole is registered for use in Lithuania. Levamisole is used sporadically, but it has been one of the most commonly used anthelmintic for many years in Lithuania (Kupčinskas et al., in press).

The information about GIN in sheep farms in Lithuania is limited and there is no data regarding AR. However, previously performed *in vivo* studies (Kupčinskas et al., in press) have showed existing problems with sheep GIN and low efficiency of anthelmintics.

The purpose of the present study was to detect the anthelmintic resistance to benzimidazoles and levamisole in sheep farms in Lithuania and compare it with results of AR in other European countries.

Methods and materials

Trial design

The survey was conducted from May 2014 to November 2014. The *in vitro* method Micro-agar larval development test (MALDT) was used for the detection of benzimidazole and levamisole resistance. Twenty-three sheep farms with regular use of anthelmintic treatments were selected for the study. Seventeen sheep farms were tested by MALDT for anthelmintic resistance (AR) to benzimidazoles and six sheep farms were tested for AR to levamisole. The sheep flocks consisted of 50–800 animals on each farm. Lambs or yearlings that had not received any anthelmintic treatment for at least 10 weeks before the initiation of the tests were examined. On each farm, faecal samples from the rectum of 15–20 animals were randomly taken. Pooled faecal samples weighing 50–100 g were stored anaerobically in plastic tubes filled with water at room temperature and processed in two days (Dolinska et al., 2014). Nematode eggs were collected by sieving the faeces through three stacked sieves with apertures of 250, 100 and 20 µm, respectively. The material collected on the 20 µm sieve was washed with water and centrifuged at 1500 rpm for 2 minutes. After that, the trichostrongylid eggs were recovered by the salt flotation method and used for *in vitro* MALDT (MAFF, 1986; Varady et al., 2006).

*Micro-agar larval development test (*MALDT)

The MALDT described by Coles et al. (2006) was used. Tests were performed in 96-well microtiter plates. For MALDT thiabendazole (TBZ) and levamisole (LEV) were used. To produce 12 final concentrations ranging from 0.0006 to 1.28 µg/ml^{-1} for thiabendazole and from

0.0156 to 32 µg/ml^{-1} for levamisole stock drug solutions of thiabendazole were serially diluted 1:2 with dimethyl sulfoxide (DMSO) and of levamisole with deionised water. Subsequently, 12 µl of the stock solutions with different final concentrations were mixed with 150 µl of 2 % Bacto agar (Difco, USA) and kept at +4°C for 5 min. Then, 10 µl of eggs (final number of eggs per well was 50) in a 0.3 mg/ml^{-1} solution of amphotericin B, to inhibit fungal growth (Sigma-Aldrich, Germany), were mixed with 10 µl of yeast extract and then added to the agar (Varady et al., 2006; Dolinska et al., 2014). Only DMSO (1.3 %) was used in the control wells. Yeast extract was prepared as described by Hubert and Kerboeuf (1984) – 1 g of yeast extract in 90 ml of 0.85 % NaCl was autoclaved for 20 minutes at 121 C˚. After that, 27 ml of this solution were mixed with 3 ml of 10x concentrated Earle's solution). The plates were incubated for seven days at 27°C. Incubation was terminated by adding Lugol's iodine solution into each well. After incubation, the proportion of unhatched eggs, L_1-L_2 and the third-stage (L3) larvae at each concentration was determined under an inverted stereomicroscope.

Data analysis

Instead of using the conventional threshold values (LC_{50} or LC_{99}), the threshold discriminating concentration was used. Farms were classified as resistant, when L_3 larvae were found on concentration 0.04 µg/ml^{-1} for thiabendazole and 2 µg/ml^{-1} for levamisole (Dolinska et al., 2012; Dolinska et al., 2013). As an additional information indicator a minimum inhibitory concentration (MIC) was checked, which is a minimum concentration required to completely inhibit development of larvae to the L_3 stage over the incubation period (Varady et al., 2006).

The study was conducted in compliance with Lithuanian animal welfare regulations (No. B1-866, 2012; No. XI-2271, 2012) and was approved by the Lithuanian Committee of Veterinary Medicine and Zootechnic Sciences (Protocol No.07/2010).

Results

A total 23 sheep farms were investigated. No resistance to thiabendazole (TBZ) were found on five farms (29.4 %). Benzimidazoles resistant gastrointestinal nematodes were found on 12 farms (70.6 %). Three (25 %) farms had high level of resistance, three farms had a medium level of resistance (25 %) and six (50 %) farms had a low level of resistance. In addition, on one farm the level of developed L_3 larvae on the concentration 0.04 µg.ml^{-1} for thiabendazole was very high – 79.5 % (Fig. 1).

No resistance to levamisole was found on 4 farms (66.6 %). Resistance to levamisole with low percentages (<3.6 %) of developed L_3 larvae was found on two farms (33.4 %).

Figs 2 and 3 summarize the results of minimum inhibitory concentration (MIC) values, obtained by MALDT. Based on MIC values, thiabendazole resistance is advanced on nine (52.9 %) farms (MIC ≥0.08 µg/ml^{-1}), levamisole resistance on two (33.4 %) farms (MIC≥4 µg/ml^{-1}).

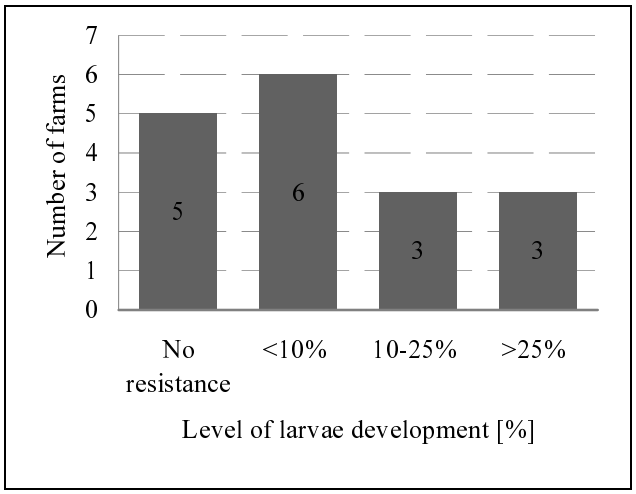

Fig. 1. **Number of sheep farms with the development of larvae at a threshold of 0.04 µg.ml-1 thiabendazole in MALDTs; 17 farms**

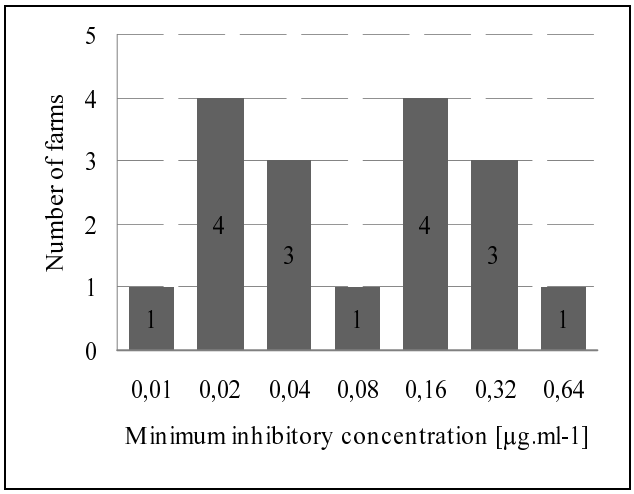

Fig. 2. **Comparison of thiabendazole MIC values in 17 sheep farms**

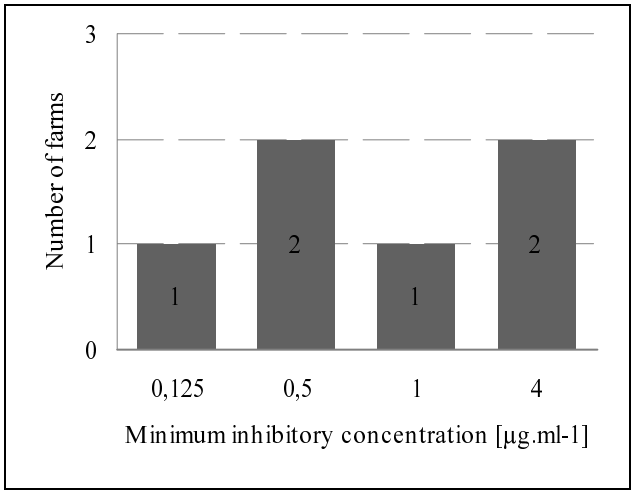

Fig. 3. **Comparison of levamisole MIC values in six sheep farms**

Discussion and conclusions

These *in vitro* surveys indicated that resistance against benzimidazoles and levamisole was present on sheep farms in Lithuania. In Europe, mainly AR to benzimidazoles and levamisole (Dolinska et al., 2014) has been observed. In our study, AR to benzimidazoles was found on 12 (70.6 %) farms out of 17. In other European countries, AR to benzimidazoles was found on 83.0 % of flocks in western France (Chartier et al., 1998), 100 % in Slovak Republic (Dolinska et al., 2014) and 13.6 % in Spain (Alvarez-Sanchez et al., 2006). These results show that there is a problem of AR in sheep in Lithuania. By *in vivo* studies, AR to fenbendazole was 20 % and one farm was suspected (6.7 %) (Kupčinskas et al., in press). In this study, we mostly investigated farms which already have problems with low efficacy of anthelmintics. That, probably, increased a general view of AR to benzimidazoles in Lithuania. AR to levamisole found on two farms (33.4 %) out of six. In these two farms, the development of L_3 larvae in concentration 2 $\mu g/ml^{-1}$ was very low: 1.7 and 3.6 %. Only six farms were investigated to determine an AR to levamisole, because few farmers are still using this anthelmintic in Lithuania. In Europe, AR to levamisole was 40 % in the United Kingdom (Taylor et al., 2009) and 100 % in Italy (Traversa et al., 2007). The AR to levamisole in Lithuania is low compared to other countries.

Data could be analysed using the conventional threshold values LC_{50} or LC_{99} (Waller et al., 1985). In MALDT using the LC_{99} criterion and the threshold discriminating concentration have the potential to detect a low level of resistance (Varady et al., 2007). The LC_{50} criterion is not able to provide early detection during the development of resistance (Dolinska et al., 2014). The way to analyse data using the threshold discriminating concentration 0.04 $\mu g/ml^{-1}$ for thiabendazole and 2 $\mu g/ml^{-1}$ for levamisole was faster, simpler and cheaper (Dolinska et al., 2012; Dolinska et al., 2013).

Furthermore, MIC values were checked as an additional information indicator to determine how far the resistance is advanced. The results indicated that almost in all farms where resistance was present it was advanced as well.

The present study for the first time shows a high prevalence of AR to benzimidazoles and a low prevalence of AR to levamisole in sheep farms of Lithuania. Further investigations are needed to prepare a proper strategy and preventive measures to avoid a faster development of AR in sheep farms in Lithuania.

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