

## Original papers

# The first reported case of resistance of gastrointestinal nematodes to benzimidazole anthelmintic in goats in Poland

Marcin Mickiewicz<sup>1</sup>, Michał Czopowicz<sup>1</sup>, Paweł Górski<sup>2</sup>, Jarosław Kaba<sup>1</sup>

<sup>1</sup>Laboratory of Veterinary Epidemiology and Economics, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, ul. Nowoursynowska 159c, 02-776 Warsaw, Poland

<sup>2</sup>Division of Parasitology and Invasiology, Department of Preclinical Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, ul. Ciszewskiego 8, 02-787 Warsaw, Poland

Corresponding Author: Michał Czopowicz; e-mail: mczopowicz@gmail.com

**ABSTRACT.** Fecal egg count reduction (FECR) test with albendazole and egg hatch test (EHT) with thiabendazole (TBZ) were performed in a dairy goat herd suspected of anthelmintic resistance to benzimidazoles. The herd had been regularly dewormed with fenbendazole for 5 previous years and despite that it remained infected with several species of gastrointestinal nematodes (*Trichostrongylus colubriformis*, *Teladorsagia circumcincta*, and *Haemonchus contortus*). Albendazole was administered *per os* at dose of 20 mg/kg to 10 goats (treated group), while 10 other goats remained untreated (control group). Fecal egg count (FEC) was determined using McMaster egg counting method before and 7 days after the treatment in the treated group, and once (at the latter moment) in the control group. EHT was performed on the pooled rectal sample collected from treated goats. EHT comprised the negative control and 7 consecutive concentrations of TBZ (0.05, 0.1, 0.2, 0.3, 0.5, 1.0, 2.0 µg/ml) according to the standard procedure. Two hundred eggs/larvae were counted to determine percentage of unhatched eggs, which was adjusted by the natural mortality. TBZ dose effective in preventing hatching of 50% of eggs (ED<sub>50</sub>) was determined using the log-probit transformation. Median FEC (range) before the treatment was 1000 (250–3450) epg in the treated group and dropped to 150 (50–500) epg after the treatment (p=0.005). Median FEC (range) after the treatment was also significantly lower in the treated than in control group (p=0.009), where it was 725 (0–5050) epg. FECR between the treated and control group was 81% (95% CI: 49%, 93%). FECR in the treated group was 83% and 74% based on average and individual approach, respectively. ED<sub>50</sub> value of TBZ was 0.78 µg/ml. Only *H. contortus* persisted in the treated group after treatment. The results indicate resistance of *H. contortus* to a benzimidazole anthelmintic, which is the first such case reported in Polish goats.

**Key words:** aggregation parameter, albendazole, egg hatch test, fecal egg count reduction test

## Introduction

Infections with gastrointestinal (GI) nematodes in small ruminants cause both subclinical and clinical diseases and result in considerable economic losses. Their control mostly relies on the use of three basic classes of anthelmintics: benzimidazoles, macrocyclic lactones (ivermectin/milbemycins), and imidazothiazoles/tetrahydroxypyrimidines, of which benzimidazoles are most commonly used due to their low price, short withdrawal period in dairy animals and wide safety margin [1,2]. Many years of their unlimited and commonly improper use have led to increasing

anthelmintic resistance, first in sheep, and then in goats all over the world [3]. Thus far resistance to benzimidazole anthelmintics in goats has been reported from most European countries, including the United Kingdom [4,5], Netherlands [6], Spain [7], Germany [8], Switzerland [9], Italy [10], France [11], Norway [12], and Denmark [13]. In Poland resistance to anthelmintics has been documented in sheep [14–16], cattle, horses and pigs [15] but never in goats. In this article we present the first case of resistance to benzimidazole anthelmintics in goats in Poland.

## Materials and Methods

The study was carried out in a goat dairy herd in the southern Poland. The herd consisted of one adult male, 52 adult females and 11 kids. The animals were housed in a half-wooden-half-brick barn. They were grazed for 8–10 hours a day, from April to November on a large pasture fenced with electric wire. They did not share the pasture with any other animals but their pasture bordered on a sheep farm. Additionally, they were fed on alfalfa hay, whey bran, oatmeal and barley grit and had supplemental pure mineral blocks.

The herd was established in 2011 by purchasing several adult goats from a single herd located in the south-eastern Poland. Then, mostly own goats were raised for replacement, however several females were still purchased each year from two other Polish herds.

The owner claimed that all goats in the herd had been regularly dewormed: in years 2011–2015 once a year using fenbendazole (Fenbenat 40 mg/g,

Vetos-Farma, Poland) at a uniform dose of 200 mg per an adult goat; in 2016 twice – on May with eprinomectin 1 mg/kg spot-on (Eprizero 0.5%, Scanvet, Poland), and on August with albendazole 15 mg/kg *per os* (Valbazen 10%, Zoetis, USA). In 2017 they were dewormed on February with eprinomectin 1 mg/kg spot-on (Eprizero 0.5%, Scanvet, Poland), on May with albendazole 15 mg/kg *per os* (Valbazen 10%, Zoetis, USA), and again in July with eprinomectin 1 mg/kg spot-on (Eprizero 0.5%, Scanvet, Poland). Before each deworming in 2017 fecal samples collected directly from the rectum (rectal samples) from 10–20 goats were examined in our laboratory and fecal egg count (FEC) test constantly showed high degree of invasion with GI nematodes (*Trichostrongylus colubriformis*, *Teladorsagia circumcincta*, and *Haemonchus contortus*). *Muellerius capillaris* and *Eimeria* spp. used to be detected as well.

The latest FEC test was done on the group of 10 randomly selected adult goats on September 22<sup>nd</sup> and its results were identical to previous ones, with

Table 1. Fecal egg count (FEC) given as eggs per gram (epg) in two groups of goats (treated and control) enrolled in the study

| Goat                             | Treated group                           |                        | Goat | FEC (epg) (Day 7)     |
|----------------------------------|---|------------------------|------|-----------------------|
|                                  | Pre-treatment (3 days before treatment) | Post-treatment (Day 7) |      |                       |
| 1                                | 800                                     | 500                    | 11   | 100                   |
| 2                                | 1050                                    | 500                    | 12   | 800                   |
| 3                                | 3450                                    | 200                    | 13   | 700                   |
| 4                                | 1050                                    | 150                    | 14   | 700                   |
| 5                                | 950                                     | 50                     | 15   | 5050                  |
| 6                                | 600                                     | 50                     | 16   | 1350                  |
| 7                                | 2800                                    | 50                     | 17   | 1800                  |
| 8                                | 250                                     | 100                    | 18   | 750                   |
| 9                                | 2100                                    | 500                    | 19   | 0                     |
| 10                               | 300                                     | 150                    | 20   | 550                   |
| Mean ± SD <sup>a</sup>           | 1335 ± 1085                             | 225 ± 196              |      | 1180 ± 1458           |
| Median, IQR <sup>b</sup> (range) | 1000, 600–2100 (250–3450)               | 150, 50–500 (50–500)   |      | 725, 100–725 (0–5050) |
| Aggregation parameter (k)        | 1.5                                     | 1.3                    |      | 0.7                   |

<sup>a</sup>standard deviation; <sup>b</sup>interquartile range

>150 eggs per gram (epg) in each sample (Table 1). On this basis resistance to anthelmintics was suspected and the fecal egg count reduction (FECR) test with the control group was performed according to Coles et al. [17,18]. Briefly, after weighing each goat on a portable livestock scale albendazole (Valbazen 10%, Zoetis, USA) was administered to the aforementioned group of 10 goats (treated group) on September 25th (Day 0). The medicine was given by the owner *per os* at dose of 20 mg/kg which is recommended for goats [19]. Seven days later (October 2nd, Day 7) 5g rectal samples from these 10 goats and from 10 other adult goats (control group), not treated with any benzimidazole anthelmintic for preceding 4 months (last time on May 10th), were examined using McMaster egg counting method which had detection limit of 50 epg according to Coles et al. [17]. Larval cultures were prepared for every group by mixing 5g of feces collected from each animal on day 7 into one pool per group. After baermannization, a minimum of 100 third stage larvae (L3) from each pool were identified at the genus level following the procedure described by van Wyk and Mayhew [20].

Three methods were applied to evaluate herd FECR:

After treatment evaluation in the treated and control group [17], where  $FECR = 100\% \times (1 - T/C)$ . T and C stood for the arithmetic mean of FEC (epg) in the treated and control group, respectively.

Before and after treatment evaluation in the treated group, without the control group [21], where  $FECR = 100\% \times (1 - T_1/T_0)$ . T<sub>0</sub> and T<sub>1</sub> stood for the pre-treatment and post-treatment arithmetic mean of FEC (epg), respectively.

Before and after treatment individual evaluation in the treated group, without control group [22], where  $FECR = 100\% \times \sum(1 - iT_1/iT_0)/n$ , where iT<sub>1</sub> is post-treatment and iT<sub>0</sub> is pre-treatment FEC (epg) in a given goat from the total number of goats (n).

We assumed that true anthelmintic resistance would be present if FECR was lower than 95%. However, on the basis of results of the latest study concerning accuracy of FECR test [23], we assumed that the anthelmintic resistance could be accurately detected if FECR in this study was below 85%, excluded if FECR in this study was above 97.5%, and the range between these two figures should have been considered in this study as inconclusive. This assumption was based on four aspects: the small group size (10 goats) and high detection limit of egg counting method used (50 epg), the high

mean pre-treatment FEC (>1000 epg) and low level of aggregation of FEC across animals (i.e. quite even dispersion of FEC across goats within the group).

To confirm the results an *in vitro* assay – egg hatch test (EHT) was performed according to Coles et al. [17,18]. Pooled rectal samples from 10 goats from the treated group were collected by the farmer on October 23<sup>rd</sup> and sent by post in 100 ml screw-top plastic bottle filled with tap water to prevent egg development. The sample arrived at laboratory and was proceeded on the next day. Eggs were extracted by sieving, centrifugation, and flotation in saturated sodium. Then, they were suspended in deionized water so that 1 ml contained at least 150 eggs, inspected under microscope to ensure that hatching had not yet begun, and placed in 16 wells of 24-well tissue culture plate (SARSTEDT, Poland). Next, thiabendazole (Sigma-Aldrich T8904, Merck, Poland; TBZ) was added to 7 consecutive pairs of wells at increasing concentrations of 0.05, 0.1, 0.2, 0.3, 0.5, 1.0 and 2.0 µg/ml, with one pair left as a negative control. The plate was sealed to prevent drying out and incubated at 25°C for 48 h, and then stained with 2 drops of Lugol's iodine per each well. Two hundred unhatched eggs and hatched larvae were counted in each well at 40× microscope magnification and the figures were averaged for each pair of wells. Then, the percentage of unhatched (inhibited) eggs was calculated and corrected for natural mortality from control wells (corrected percentage inhibition, thenceforth referred to as cPI). TBZ concentrations were log transformed and the S-shaped dose-response curve was fitted by transforming cPI to their probits, defined as normal equivalent deviates (area under the standard normal curve to the left from the position on the curve corresponding to the probability equal to a given cPI) increased by 5 to avoid calculating with negative numbers [24]. The log-probit transformation was used to determine TBZ concentration which inhibits hatching of 50% of eggs (effective dose, ED<sub>50</sub>). TBZ discriminating dose (i.e. concentration which is expected to prevent hatching 99% of eggs) of 0.1 µg TBZ/ml was assumed [18] and benzimidazole resistance was considered as confirmed if the ED<sub>50</sub> value was above the discriminating dose [17].

Numerical variables were non-normally distributed according to the Shapiro-Wilk test. Therefore, they were presented as the median, interquartile range (IQR) and the range, and

Table 2. The results of the egg hatch test based on counting 200 eggs/larvae per well

| Thiabendazole concentration<br>(µg/ml) | Average number of     |                         | Corrected percentage of<br>inhibited eggs <sup>a</sup> |
|--|-----------------------|-------------------------|--|
|  | hatched eggs (larvae) | inhibited (killed) eggs |  |
| 0 (negative control)                   | 185                   | 5                       | –  |
| 0.05                                   | 179                   | 16                      | 6%   |
| 0.1 <sup>b</sup>                       | 171.5                 | 28.5                    | 12%  |
| 0.2                                    | 145                   | 55                      | 26%  |
| 0.3                                    | 101                   | 99                      | 48%  |
| 0.5                                    | 121                   | 79                      | 38%  |
| 1.0                                    | 89.5                  | 110.5                   | 54%  |
| 2.0                                    | 69                    | 131                     | 65%  |

<sup>a</sup>correction for the natural mortality in the control wells; <sup>b</sup>thiabendazole discriminating dose

compared between the treated and the control group using the Mann-Whitney U test, and between paired observations using the Wilcoxon signed-rank test. All tests were two-tailed and a significance level ( $\alpha$ ) was set at 0.05. FEC was also presented as the arithmetic mean and standard deviation (SD) since these figures were used in the calculation of FECR. Level of aggregation of FEC across animals was given by the aggregation parameter  $k = (\text{mean FEC}^2 - \text{SD}^2 / \sqrt{\text{group size}}) / (\text{SD}^2 - \text{mean FEC})$  [25]; the lower  $k$  the more aggregated (clumped) FEC in single animals in a group; the higher  $k$  the closer FEC distribution to even (random) distribution.

All analyses were performed in Statistica 12 (StatSoft, Inc.) and Excel® Microsoft® 2013.

## Results

The goats enrolled in the FECR test were either born in the herd (6 in the treated group and 7 in the control group) or purchased and introduced in 2012 (4 and 3 goats, respectively). Goats' age ranged from 6 months to 9 years with the median of 4.5 years (IQR from 5 to 9 years) and did not differ between the treated and control group ( $p=0.520$ ).

FEC in the treated group before treatment did not differ significantly from FEC in the control group ( $p=0.405$ ). The aggregation parameter indicated low level of aggregation in both groups (Table 1).

Treatment resulted in significant reduction of FEC in the treated group ( $p=0.005$ ) and FEC after treatment in the treated group was significantly

lower than in the control group ( $p=0.009$ ) (Table 1).

FECR between the treated and control group (method 1) was 81% (95% CI: 49%, 93%). FECR in the treated group was 83% according to method 2, and 74% according to method 3. All three results indicated resistance to benzimidazole anthelmintic.

The pooled rectal sample examination performed in the control group and in the treated group before treatment showed presence of *T. colubriformis*, *T. circumcincta*, and *H. contortus*, whereas only *H. contortus* was still present in the treated group after treatment.

The discriminating dose of 0.1 µg TBZ/ml prevented hatching of only 12% of eggs (Table 2). ED<sub>50</sub> value was 0.78 µg/ml.

## Discussion

Our study shows for the first time that resistance of some species of gastrointestinal nematodes (namely *H. contortus*) to benzimidazole anthelmintics occurs in goat population in Poland. To increase reliability of the study we decided to apply three different approaches to evaluating FECR: the classical with the control group and alternative without the control group based either on average or individual FEC (i.e. FECR<sub>1</sub>, FECR<sub>3</sub> and iFECR<sub>3</sub>, respectively, according to Cabaret and Berrag [22]). The former two methods showed similar FECR, which was in turn lower in the latter one. This is in line with observations of Cabaret and Berrag [22], who argued in favor of the individual approach by

presenting the discrepancies between average FECR (indicating susceptibility), and egg hatch assay or genotyping of benzimidazole resistant strongyles in some previous studies. Whatever the truth is all three methods we used showed anthelmintic resistance according to criteria much more stringent than classically applied. Using by default one common cut-off of FECR of <95% for all methods seems controversial as it does not take into account different properties of animal groups enrolled and different study designs. Levecke et al. [23] performed simulations which provided more realistic cut-offs and refined the interpretation of FECR tests by introducing the “grey zone” of inconclusive results. We believe that assuming the more strict cut-off is crucial and considerably increases the trustworthiness of our results.

There are several reasons which make goats in Poland highly prone to the development of anthelmintic resistance. First, goats are known to eliminate various medicines quicker than sheep due to differences in liver metabolism, which results in higher doses needed to ensure anthelmintic efficacy [26,27]. Unfortunately, this fact is not commonly known in Poland and ovine doses are frequently extrapolated to goats. Secondly, weighing goats before deworming is far from being the routine practice in the field. As many owners perceive goats as thin even if they are in fact obese, body weight is likely to be underestimated. Both the aforementioned facts lead to underdosing of anthelmintics. On the other hand, fecal examination is rarely done before anthelmintic treatments, which likely results in many redundant anthelmintic treatments. Moreover, goats are kept mostly as dairy livestock in Poland and long withdrawal periods of some anthelmintics substantially narrow their range down mainly to benzimidazoles. As benzimidazoles are also the least expensive anthelmintics, they tend to be repeated for many consecutive years (which was the case also in the herd described in our study). In view of the aforementioned favorable circumstances we are surprised that anthelmintic resistance in goats has not been detected earlier, even though we have carried out parasitic monitoring in many Polish goat herds for last years.

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