



# Anthelmintic resistance in gastrointestinal nematodes in sheep raised under mountain farming conditions in Northern Italy

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## ABSTRACT

Anthelmintic resistance (AR) in sheep raised under mountain farming conditions in South Tyrol (Northern Italy) was assessed on eight farms (n=99 animals). A faecal egg count reduction (FECR) test was done after routine anthelmintic treatments. Furthermore, on 27 farms (n=306 animals), a FECR test was conducted after oral formulations of a macrocyclic lactone (ML), benzimidazole (BZ) (partly in combination with salicylanilide (SA)) or a combination of imidazothiazole and SA were applied under controlled conditions on the same farm. Following routine treatments, three of five ML-treated flocks showed an adequate efficacy, while the other two reached a FECR of only around 75 per cent. A wide range of gastrointestinal nematode genera were identified in one flock following the treatment. From the three BZ-treated flocks, only one showed an adequate FECR, both other farms reached 68 per cent and 84 per cent, respectively. Under controlled conditions, FECR ranged between 77 per cent and 81 per cent indicating AR for all the applied anthelmintics. *Trichostrongylus* species, *Teladorsagia* species and *Haemonchus* species were identified after ML treatment, *Teladorsagia* species after BZ treatment and *Trichostrongylus* species and *Haemonchus* species after combined BZ and SA application. Taking into consideration that underdosing might have affected results of the routine treatments, a high prevalence of AR was found in sheep under mountain farming conditions.

## INTRODUCTION

Endoparasites cause great losses in small ruminant production through reduced weight gains, decreased milk yields, discarded organs at slaughter and even deaths.<sup>1</sup> Regular whole-flock treatments with anthelmintics is still the most commonly used measure to control endoparasitic infections in small ruminants. However, their decreasing efficacy because of its regular use has gained interest, and anthelmintic resistances (ARs), especially of gastrointestinal nematodes, in small ruminants are proven in numerous countries.<sup>2-6</sup>

Under mountain farming conditions, the commonly practised alteration between pasture areas at lower altitudes in spring and

autumn and communal summer pastures at high altitudes beside a barn period without access to pasture in winter might impact parasitological infections and the development of AR. Compared with other larger scale production conditions where adoption of alternative GIN control measures such as the management of refugia, rotation of pasture areas or targeted (selective) treatments by farmers is already low,<sup>7,8</sup> its implementation is even more complicated. This holds especially true for many alpine regions such as South Tyrol, Northern Italy, where small ruminants constitute an important proportion of the livestock population but are predominantly raised by small-scale or hobby farms. Recently, more than 100 small ruminant farmers in this region were surveyed.<sup>9</sup> The study reported that farmers perceive gastrointestinal nematodes as the most frequent parasites with more than 90 per cent of them applying anthelmintic treatments at least once per year with very limited alteration of anthelmintics. Additionally, there was almost no use of coprological examinations to validate efficacy, even though 14 per cent of the sheep and 18 per cent of the goat farmers already perceived that anthelmintics were not or only partly effective.

Therefore, it was the aim to assess the status of AR in sheep raised under mountain farming conditions in Northern Italy. Thereby, AR was assessed after farmers conducted routine treatments as well as under controlled conditions in which different anthelmintics were applied on the same farm.

## MATERIALS AND METHODS

The applied sampling protocols met the International Guiding Principles for Biomedical Research Involving Animals.<sup>10</sup>



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### Selection of farms

Sheep farmers of the province South Tyrol, Northern Italy (46.73° North, 11.29° East) were invited through an announcement in the local agricultural magazine in autumn 2015 to participate in this study. The study was also promoted by the South Tyrolean farmer association for small ruminants. An invitation letter described the purpose of the study, provided assurance of confidentiality, asked for permission to publish the anonymous responses and the willingness to participate in the evaluation of the anthelmintic efficacy. In total, the questionnaire consisted of 32 closed and 3 open questions on farm management, system, herd size, breeds, other livestock on the farm, farm sizes, elevations and management of pastures, drenching practices including the choice of anthelmintics, application practices, rotation of anthelmintics and the perceived efficacy and side effects. Details are described by Lambertz and others.<sup>9</sup> From this dataset of 68 sheep flocks, eight farmers agreed to participate in a faecal egg count reduction (FECR) test after conducting their routine anthelmintic treatments. Furthermore, 27 sheep farmers agreed to participate in an FECR test under controlled conditions, in which three different anthelmintics of a macrocyclic lactone (ML), benzimidazole (BZ) (partly in combination with salicylanilide (SA)) or a combination of imidazothiazole (IT) and SA were applied on the same farm. The recommendations by the World Association for the Advancement of the Veterinary Parasitology regarding the detection of AR in nematodes were followed.<sup>11 12</sup> The FECR test was performed once per farm in autumn 2015, spring 2016 and autumn 2016. Data on farm management, including husbandry system, herd size, breeds, other livestock on the farm and sizes, elevations and management of pastures were collected using the questionnaire as described above.

### FECR test of routine anthelmintic treatments

Farmers applied their routine treatments against gastrointestinal nematodes using commercial anthelmintics selected by their attending veterinarians following the manufacturers' instructions for dosage. As a precondition for participating in this study, the animals must not be treated with anthelmintics within the previous three months. The entire flocks were treated, and all animals were naturally infected with GIN. Farmers and attending veterinarians were not advised to select specific anthelmintics. Individual faeces samples were taken prior and 10–14 days after anthelmintic application. Only individuals older than three months were sampled.

### FECR test under controlled anthelmintic treatments

Prior to the application of anthelmintics, animals were weighed individually to calculate the correct dosage. Animals were randomly allocated to one of four groups at each farm. In addition to an untreated control group, the other three groups were treated with oral formulations of a ML, BZ (partly in combination with SA) or a combination of IT and SA. In detail, ivermectin (ML,

Oramec, 0.8 mg/ml, 0.25 ml/kg bodyweight; Merial Italia S.p.a.), netobimin (BZ, Hapadex, 50 mg/ml, 0.15 ml/kg bodyweight; MSD Animal Health S.r.l.), fenbendazole (BZ, Panacur, 25 mg/ml, 0.2 ml/kg bodyweight; MSD Animal Health S.r.l.), a combination of oxfendazole (BZ) and closantel (SA) (Oxydrench, 25 mg/ml oxfendazole, 50 mg/ml closantel, 0.2 ml/kg bodyweight; Bayer S.p.A) and a combination of levamisole (IT) and oxcyclozanide (SA) (Toloxan, 12.73 mg/ml levamisole, 30 mg/ml oxcyclozanide, 0.3 ml/kg bodyweight; Azienda Terapeutica Veterinaria S.r.l) were applied.

Individual faeces samples were taken prior and 14 days after anthelmintic application. The age of the animals was recorded during faeces sampling and body condition score (BCS) was scored on a 1–5 scale according to Jefferies.<sup>13</sup> Treatments were conducted either before the animals were moved to alpine pastures in spring 2016 (11 farms) or after they returned from alpine pastures in autumn 2016 (16 farms).

### Parasitological measurements

Fresh faecal samples were directly collected from the rectum of the individual animals. After collection, the samples were stored cool during transportation to the laboratory and then stored at 4°C in the refrigerator until analysis to avoid hatching of the eggs. Samples prior and after treatments for calculation of FECR were available from 77 sheep after routine treatments and from 306 animals after controlled treatments. Faecal egg counts (FECs) were done using a modified McMaster method<sup>14</sup> with 60 ml of saturated NaCl solution as the flotation fluid (specific gravity=1.2) and 4 g of faeces to determine eggs per gram of faeces. Each egg counted represents 50 eggs per gram of faeces. According to Cabaret and Berrag,<sup>15</sup> FECR was calculated using the following formula, in which each host served as its own control:

$$FECR = (1/n)\Sigma(100x(1-([T_{i2}-T_{i1}])))$$
 where  $T_{i2}$  is post-treatment and  $T_{i1}$  is pretreatment FEC in host  $I$  from a total of  $n$  hosts. Animals with a negative FEC (<50 eggs per gram) at the first sampling were excluded from the calculation of FECR. For further identification of nematode species, third-stage larvae (L3) were cultured with pooled faeces (10–20 g). For farms that participated in routine treatments, one sample was pooled per flock. Under controlled anthelmintic treatments, one sample was prepared per treatment group. Separate cultures were prepared for the farms sampled in spring and autumn 2016. Results are presented as mean of these two samples. L3 were recovered from the coprocultures by applying the Baermann technique.<sup>14</sup> The first 100 randomly selected L3 of each sample were identified as *Teladorsagia* species, *Trichostrongylus* species, *Oesophagostomum* species, *Chabertia* species, *Haemonchus* species, *Bunostomum* species and *Cooperia* species by microscopy.<sup>14</sup> The percentage of larval type was calculated based on the counted L3 when fewer than 100 L3 were isolated from a sample.

## Statistical analysis

The statistical analysis was performed with SAS statistical package V.9.3. Results of the routine treatments and controlled treatments were analysed separately. The MEANS procedure was used to calculate arithmetic mean and SD for FECs before and after treatments as well as arithmetic mean and 95 per cent CIs of FECR. AR was assumed if FECR was <95 per cent and the lower 95 per cent CI was <90 per cent.<sup>12</sup> If only one of these criteria were met, AR was considered as suspected. For the controlled treatments, AR against multiple anthelmintics on the same farm was calculated at farm level applying the same thresholds.

In order to estimate the effect of age class and BCS on the risk of AR, a logistic regression analysis was performed with the GLIMMIX procedure for the flocks that were treated under controlled condition. FECR was transformed into a binary variable where class 0 represented an adequate FECR of >95 per cent and class 1 of <95 per cent and thus the risk for lack of anthelmintic efficacy. The following age classes were grouped: <6 months, 6–12 months, 1–2 years, 2–3 years, 3–4 years and >4 years. Farm was included as random effect, and results are presented as OR and 95 per cent CI.

## RESULTS

### FECR test of routine anthelmintic treatments

The average flock size of the eight farms that were included in the FECR test of routine anthelmintic treatments was 27 (range 13–52). One farm raised the animals for milk production, the others for meat production. Three flocks were composed of brown mountain sheep, two of Friesian milk sheep, one of Jura sheep, one of Villnoesser sheep and one of Tirolean mountain sheep. One farm was certified as organic farms. Only one farm was managed in full time, while the others in part-time. Animals of all farms had access to pasture at least from April until October and grazed on communal pasture areas at altitudes above 1500 m during summer months. Seven farmers perceived GIN to occur regularly on their farm. Four farmers conducted whole-flock anthelmintic treatments twice per year, one during the dry-off period and two when clinical signs, such as diarrhoea or emaciation, were noticed. Two farmers perceived previous treatments not to be effective, whereas only one farmer used coprological analysis for efficacy control.

Results of the FECR test are presented in table 1, whereas five farms applied ML (subcutaneous) and three farms BZ (oral) at the recommended dosage of the manufacturer. The arithmetic mean FEC prior to the treatments ranged between 356±261 and 1125±860, with *Haemonchus* species and *Trichostrongylus* species being the most prevalent genera. In three out of five flocks treated with ML, adequate efficacy was recorded, while the other two ML-treated flocks reached a FECR of only around 75 per cent. After treatment, a wide range of GIN genera were identified in one of these flocks. From the

**Table 1** Applied anthelmintics, arithmetic mean±SD of faecal egg counts (FEC) before and 10–14 days after treatment, arithmetic mean and 95 per cent CI of faecal egg count reduction (FECR) (%) and larval identification (LI) before and after routine anthelmintic treatments of eight sheep flocks

Farm	n	Anthelmintic (class)	Pre-FEC (mean±SD)	Post-FEC (mean±SD)	FECR mean	95 per cent CI	LI (pretreatment/post-treatment)			
							Tr	Te	Ha	Other
1	6	Ivermectin (ML)	1125±860	50±122	98.2	94 to 100	22/0	9/0	67/100	2 Co/0
2	12	Ivermectin (ML)	412±391	0	100.0		0/*	0/*	100/*	0/*
3	4	Ivermectin (ML)	760±535	0	100.0		0/*	0/*	100/*	0/*
4	18	Ivermectin (ML)	633±416	135±153	73.9	56 to 91	48/23	0/0	18/51	6 Co, 11 Bu, 16Oe/9 Co, 16 Bu
5	12	Moxidectin (ML)	809±844	91±80	75.7	56 to 95	35/-	7/-	57/-	0/-
6	4	Albendazole (BZ)	809±276	24±28	96.1	89 to 100	46/0	15/100	39/0	0/0
7	7	Albendazole (BZ)	920±564	317±371	68.4	40 to 97	51/0	0/0	38/100	11 Co/0
8	26	Netobimin (BZ)	356±261	71±171	84.4	72 to 97	47/100	0/0	41/0	12 Bu/0

- no larvae in culture.

\*Not done, because of 100 per cent FECR.

Bu, *Bunostomum*; BZ, benzimidazole; Co, *Cooperia*; Ha, *Haemonchus*; ML, macrocyclic lactone; n, number of animals; Oe, *Oesophagostomum*; Te, *Teladorsagia*; Tr, *Trichostrongylus*.

**Table 2** Results of the faecal egg count reduction (%) test and larval identification (LI) before and 14 days after anthelmintic treatments of 27 sheep flocks

Anthelmintic (class)	n	Pre-FEC (mean±SD)	Post-FEC (mean±SD)	FECR mean	95 per cent CI	LI (pretreatment/post-treatment*)			
						Tr	Te	Ha	Other
Control	35	423±376	200±261			67/54	13/24	15/17	2 Co, 1 Bu, 1 Oe/ 5 Co
Ivermectin (ML)	92	360±372	95±221	80.8	74 to 88	62	7	31	0
Netobimin (BZ)	48	700±1341	224±672	76.8	66 to 88	0	100	0	0
Oxfendazole (BZ)+closantel (SA)	59	417±691	116±560	79.5	70 to 89	66	0	34	0
Levamisole (IT)+oxyclozanide (SA)	72	423±540	135±374	78.6	70 to 87	–	–	–	–

– no larvae in culture.

\*For treated groups LI post-treatment are presented only.

Bu, *Bunostomum*; BZ, benzimidazole; Co, *Cooperia*; Ha, *Haemonchus*; IT, imidazothiazole; ML, macrocyclic lactone; n, number of animals; Oe, *Oesophagostomum*; SA, salicylanilide; Te, *Teladorsagia*; Tr, *Trichostrongylus*.

three BZ-treated flocks, only one showed an adequate FECR of >95 per cent. Both others showed an efficacy of 68 per cent and 84 per cent, respectively. In one flock, only *Trichostrongylus* species, in one *Haemonchus* species and in the other *Teladorsagia* species were identified after treatment.

#### FECR test under controlled anthelmintic treatments

The average flock size was 27 (range 4–90). Animals on all farms were raised for meat production, and the breed was Tirolean mountain sheep. Three farms were certified as organic farm. All farms were managed in part-time. Animals of all, except three, farms had access to pasture at least from April to October and grazed on communal pasture areas at altitudes above 1500 m during summer months. Sixteen farmers used two whole-flock treatments per year and four one per year, three farms treated after coprological analysis and two when clinical signs, such as diarrhoea or emaciation, became obvious. Three farmers perceived previous treatments not to be effective, whereas neither of the farmers used coprological analysis for efficacy control.

The untreated control animals in sheep flocks had an average FEC of 423±376 at the first sampling and of 200±261 at the second, with a wide range of GIN larvae identified at both samplings (table 2). Treated groups had an average FEC ranging between 360±372 and 700±1341 before anthelmintics were applied. Post-treatment FECs were reduced in all groups, but FECR ranged only between 76.8 and 80.8 indicating AR for all the applied anthelmintics. Larvae identified after sheep were treated with ivermectin (ML) were composed of *Trichostrongylus* species, *Teladorsagia* species and *Haemonchus* species, while those in the group treated with netobimin (BZ) were *Teladorsagia* species and those treated with oxfendazole (BZ)+closantel (SA) were identified as *Trichostrongylus* species and *Haemonchus* species. In 10 of the 27 farms, AR to more than one anthelmintic was observed.

The ORs for the age classes and BCS for the risk of FECR <95 per cent are presented in table 3. Animals in the different classes below 4 years of age had a ratio below 1 when compared with the reference class (>4 years), so that they were at a lower risk for FECR <95 per cent. One-fifth of the animals were observed with a BCS of 2, and these had an OR of 0.565 when compared with the reference, which were animals with a BCS of 3.

#### DISCUSSION

The aim of this study was to evaluate the occurrence of AR in sheep after routine anthelmintic treatments with commonly used drug formulations (eight flocks) and after controlled treatments applying different anthelmintics on the same farm (27 flocks). The study was conducted under the specific conditions of mountain farming. Alternatives to the use of veterinary drugs such as selective treatments<sup>16 17</sup> in order to reduce the risk of the development of AR are far from being adopted in many

**Table 3** OR estimates of the logistic regression of age class and BCS on the risk of FECR <95 per cent 14 days after anthelmintic treatments of 27 sheep flocks

Factor	n	OR	95 per cent CI
Age class			
<6 months	45	0.238	0.080 to 0.707
6–12 months	24	0.063	0.007 to 0.538
1–2 years	43	0.329	0.105 to 1.029
2–3 years	60	0.442	0.175 to 1.114
3–4 years	51	0.223	0.077 to 0.650
>4 year	48	1	
BCS			
2	54	0.565	0.233 to 1.372
3	217	1	

BCS, body condition score; FECR, faecal egg count reduction.



countries.<sup>18</sup> For the studied region, this was pointed out by a recent survey and prevalence study involving more than 120 sheep and goat flocks.<sup>9</sup> One major reason why the implementation of AR-limiting strategies is complicated under mountain conditions is that animals from various farms are usually grazing together on communal grazing land without regulations on the use of anthelmintic treatments being in place. If treatments are conducted, commonly whole-flock treatments once or twice annually with a very limited rotation of applied anthelmintics and without faecal sampling for efficacy control are applied.<sup>9</sup> From a farmer's point of view, whole-flock treatments are warranted, because animal care and treatments during the summer grazing period at high altitudes are further complicated. Also, farms, which are generally small scale, are most commonly run as part-time or hobby farms with very limited income generated by small ruminants, so that veterinary care is limited, too.

AR to a wide range of GIN in small ruminants were proven in a growing number of countries, such as England and Wales,<sup>19</sup> France,<sup>3</sup> Norway,<sup>4</sup> Sweden,<sup>20</sup> Denmark,<sup>5</sup> The Netherlands,<sup>21</sup> Germany,<sup>22</sup> Austria,<sup>23</sup> Switzerland,<sup>24</sup> Lithuania<sup>6</sup> and Slovakia.<sup>25</sup> In Central Italy, Traversa and others<sup>2</sup> already reported multiple ARs of gastrointestinal trichostrongylids in sheep. While Geurden and others<sup>26</sup> found a high efficacy for ML, AR was found on several farms after application of BZ and IT. Results of the present study confirm studies that proved a wide occurrence of AR in sheep in many European countries. Even though studies under conditions of mountain farming are very limited, the risk for AR must be considered high due to the specific farming conditions mentioned above. Farms that were assessed after routine treatments applied either ML or BZ. Results of the routine treatments must be interpreted with caution taking into account bias caused by underdosing as farmers dosed based on body weight estimation. The fact that ML was effective in 3/5 farms only partly agrees with the high efficacy found in Italy by Geurden and others<sup>26</sup> and may be partly explained by the prominent use of MLs in the study region.<sup>9</sup> The finding that two out of three BZ treatments lacked efficacy, however, widely agrees with the mentioned study from Italy. While *Teladorsagia* species was the most common GIN larva identified in post-treatment cultures by Geurden and others,<sup>26</sup> it was a wide range of larvae in the ML-treated farms with observed AR and *Trichostrongylus* species in the BZ-treated flock.

Referring to the small-scale structure of the farms where the problem of AR development is generally not properly addressed, veterinarians are the key persons to give advice on the correct drug use. It is important that veterinarians also focus more on the alteration of anthelmintic classes, especially because farmers already experienced a lack of efficacy of previous treatments. The available studies on the prevalence of AR were usually conducted under controlled conditions, so that comparable results under routine conditions as assessed in this study are not available to the authors' knowledge yet. Consequently,

factors such as an incorrect dosage because of wrong estimations of animal weights to calculate the dosage, which may be prominent under practical, especially small-scale farming conditions, are not considered.

The study design to test AR under controlled conditions applying different oral formulations on the same farm is generally compliant with the recommendations made by Coles and others.<sup>11</sup> Recently, these criteria were confirmed to be the most appropriate for classifying drug efficacy.<sup>27</sup> Even though post-treatment sampling is proposed at 14 days after treatment when multiple drugs are tested, this may not be ideal for the BZ-treated and IT-treated groups. Given the fact that farms in the studied region are of small scale, the number of animals sampled per farm was partly low. Also, animals to be included in the study were not selected for high individual pretreatment FEC values, and treatment groups were not blocked by pretreatment FEC, because sheep from the different farms were treated when herded prior or after being moved to/from alpine summer pastures. Nevertheless, valid conclusions can be drawn for the various applied anthelmintics from the total number of animals with data on FECR and given the observed pretreatment FEC levels. Because study farms were not selected based on the potential risk for AR, results can be assumed to represent the true prevalence of AR in the study region. Arithmetic means were used instead of geometric ones to avoid bias in calculating efficacy.<sup>28</sup>

Results of the treatments conducted under controlled conditions clearly undermine findings of the routine treatments. AR was found against all the applied anthelmintic classes, with all *Trichostrongylus* species, *Teladorsagia* species and *Haemonchus* species being found in post-treatment larval cultures. Even the newly developed combinations of BZ and IT together with SA did not reach an adequate efficacy. To the authors' knowledge, this is the first report in which the risk for FECR to be <95 per cent was assessed based on age and BCS. Even though animal numbers were not equally distributed by age class, the risk for lack of efficacy was lower in all age classes in sheep <4 years. Regarding the body condition, animals with a lower BCS, thus representing animals prone for suspected GIN infection, were at lower risk compared with animals with better BCS. Further studies are, however, needed to validate these findings.

Clearly, measures to counteract the further spread of AR are needed when small ruminants are managed under the specific conditions of mountain farming where animals from various farms spent the summer months together on communal alpine grazing areas. Because the implementation is complicated as farms are generally managed in part-time with limited veterinary care, instructions on the correct application of anthelmintics and especially on the measures to reduce the spread of AR, that is, alteration of anthelmintics, must be adopted immediately. As anthelmintic treatments will remain the predominant measure to control GIN infections in small ruminants in the near future, targeted selective

treatments to maintain refugia of susceptible GIN should be considered by farmers and veterinarians alike, given the lack of efficacy proven in this and many other regions. The use of coprological analysis to identify individual animals which need to be treated, however, must be considered a major challenge, especially under the specific conditions of mountain farming. Nevertheless, regular scoring of BCS together with the use of faeces sampling, may be a starting point, which is feasible for a wide range of farmers and veterinarians.

## CONCLUSION

This first report on the prevalence of AR in sheep in the mountainous region of South Tyrol reveals a high prevalence of AR against the most commonly used anthelmintics in the region. Though the implementation of alternative measures to control GIN infections under mountain farming conditions may be further complicated, the lack of efficacy of treatments as assessed in this study after routine treatments and under controlled conditions demand immediate actions. However, bias caused by underdosing at routine treatments must be considered. As a first step, a correct application, that is, dosage, and alteration of available anthelmintics must be ensured. Because anthelmintic treatments will remain the predominant measure to control GIN infections in the near future, targeted selective treatments may be a further step to reduce the risk of AR development.

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