

RESEARCH PAPER

Rampancy of Antinematicidal Resistance Among Gastrointestinal Nematodes in Kurdish Goat Breeds

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ABSTRACT:

The current study was designed to determine the emergence of resistance among gastrointestinal roundworms of native goats against renowned synthetic antinematicidals such as fenbendazole, avermectin and levamisole. To meet the survey requirements, six commercial goat farms, located in Sothern and Western Erbil province-Kurdistan region of Iraq, were chosen after performing qualitative parasitological assays. The study was executed from September to end of November, 2019. From each farm, 30 adult goats were haphazardly divided into two groups, a group (n=15) for treatment and other group (n=15) served as control. The faecal egg count reduction test (FECRT) and egg hatch assay (EHA) have disclosed emergence of resistance against fenbendazole. According to RESO Computer Program, the estimated FECR% in farms 1 and 2 were 70.72 and 79.55, whilst the lower confidence intervals 95% were 63 and 73.8 respectively. The LC_{50} value of fenbendazole after conducting EHA, calculated through probit analysis, was estimated to be $2.11 \mu\text{g ml}^{-1}$ (range 1.47-2.34). Regarding avermectin, the computed FECR% in farms 3 and 4 were 93.92 and 92.98, while the lower confidence intervals 95% were 92.91 and 90.7 respectively, which signified the presence of suspicion about prevalence of resistance against avermectin. For levamisole, the calculated FECR% in farms 5 and 6 were 95.99 and 96.38, whilst the lower confidence intervals 95% were 95.1 and 95.7 respectively. Consequently, the parasitic nematodes were susceptible to this synthetic chemotherapeutic in the region.

KEY WORDS: Indigenous caprine, Alimentary tract nematodes, Synthetic dewormers, Antinematicidal resistance, Erbil province
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1.INTRODUCTION

Indisputably, infestation with gastrointestinal (GI) nematodes is deemed one of the most rampant parasitosis threatening small ruminant resources in the world particularly the Southern Hemisphere (Kaplan and Vidyashankar, 2012) and even European countries (Blake and Coles, 2007; Furgasa *et al.*, 2018). For decades, livestock raisers have relied greatly on synthetic dewormers to cure nematodiasis in ovine and caprine (Taylor *et al.*, 2007; Hamad *et al.*, 2018).

ailments have been mitigated; but on the other hand, development of antinematicidal resistance (AR) was the consequence of this practice (Gilleard, 2006).

On a particular flock of small ruminants, AR can arise in one species of GI nematodes against numerous synthetic antinematicidals or in numerous species of alimentary tract roundworms towards one type of dewormer (Chartier *et al.*, 1998). In tropical and sub-tropical zones, where AR is historically of considerable worry since last five decades, comprehensive surveys and studies have revealed that the dilemma is serious and obviously associated with recurrent deworming, widespread of *Haemonchus contortus* (a trichostrongylid helminth dwelling abomasa of sheep and goats) and the existing kind of pasture management (Waller, 1995; Kalkal *et al.*, 2019).

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The emergence and rampancy of AR seem to differ geographically according to the existing climatological conditions, species of parasitic roundworms and deworming policy followed in the area. Having said, the rate of emergence of antinematocidal-resistant individuals has commonly been slower in moderate regions in the European countries as compared to other warm geographical zones in the Southern Hemisphere (Jabbar *et al.*, 2006). In Kurdistan region, the performed studies on AR in GI nematodes of sheep and goats are very rare and the limited surveys that have been done were related to ovine nematodes (Ahmed *et al.*, 2015; Ahmed *et al.*, 2019). Hence, this comprehensive survey was encompassed the likely emergence of resistance against renowned synthetic chemotherapeutics (broad-spectrum anthelmintics) including fenbendazole, avermectin and levamisole by GI nematodes of native goats in some districts of Kurdistan region.

2. Materials and Methods

2.1. Goat farms

Six private goat flocks were chosen from the files of the local veterinarians in September- end of November 2019. The farms that underwent this survey were located in Southern (Kandinawa district) and Western (Shamamic district) Erbil province-Iraq. The size of herd, expressed as adult does (females of goats) merely, ranged from 40 to 50 on each goat farm.

2.2. Procedure

The routine procedure to diagnose AR in parasitic nematodes was that adopted by the World Association for the Advancement of the Veterinary Parasitology (W.A.A.V.P) (Coles *et al.*, 1992). None the trialed goats had been deparasited with any antinematocidal therapeutic agent for at least two months before pursuing the survey. On each of the 6 goat ranches, 30 goats, more than 1 year old, were chosen, clinically examined by the researcher (Veterinary Doctor), and arbitrarily allocated into two groups (n=15). A group was selected as untreated control and other one represented as a treatment group. The experimental animals (farm 1 and 2) were drenched fenbendazole orally by a drench gun at 10 mg/kg BW (double dose). Avermectin injected subcutaneously at 0.4 mg/kg BW (double dose) to animals in farm 3 and 4, whilst levamisole was administered orally at 12 mg/kg BW (1.5 dose) to

animals in farm 5 and 6. Dewormer doses were calculated according to the heaviest animal of each group. The reason behind giving high doses to goats is back to the difference in bioavailability and potency of aforesaid synthetic drugs between ovine and caprine (Bogan *et al.*, 1987; Sangster *et al.*, 1991).

2.3. Preliminary tests to determine natural infections with GI nematodes

●Faecal examination

Qualitative and quantitative parasitological techniques were carried out to diagnose natural infestations with different GI nematodes and during performing other steps of the survey (Coles *et al.*, 1992; Iqbal *et al.*, 2006; Radostits *et al.*, 2007).

●Coproculture

Coprocultures were executed to determine the contribution of different GI nematodes in whole natural parasite infections following MAAF (1986). Faecal specimens, collected directly from the rectum, from each group of experimental animals were pooled and cultured in plastic containers. Amphotericin B (5 µg g⁻¹) was added to prevent fungal contaminations. The cultures were incubated for seven days at 27±1°C. Then, the nematode larvae (L₃) were collected using Baermann apparatus.

●Baermann technique

This approach was conducted to collect the larvae (L₃) of GI nematodes from the coproculture. Approximately 15g of the incubated faeces were wrapped up in medical gauze and put in the Baermann apparatus funnel. Warm water was added to induce larval motility toward the end of collecting tube. The "Baermann" was left overnight and a small volume of water was collected and poured in a plastic container. Then the water specimen transferred to a petridish, Lugol's iodine was added (Iqbal *et al.*, 2006) and finally, larvae were recognized following MAFF (1986).

2.4. Studies on antinematocidal resistance

●Fecal egg count reduction test (in vivo assay)

The experimental animals (n=15) in group 1 were given fenbendazole at a dose mentioned previously; whereas group 2 served as infected untreated control (n=15) in each goat farm (two farms). Eggs per gram of faeces (EPG) were counted in control group and in fenbendazole group (group 1) after 10-14 days of treatment.

Faecal specimens were collected individually once from all the goats and applied for faecal egg counting employing Whitlock Universal Egg Counting Slide (provided by JA Whitlock & Company, PO Box 51, EASTWOOD NSW 2122 AUSTRALIA). The following formula was used to calculate EPG:

$$\text{EPG} = \frac{\text{Total eggs in chamber 1, 2 and 3}}{3} \times 50$$

(dilution factor)

The mean EPG for the treatment group was determined and compared with that of the control group. For this purpose, Faecal egg count reduction percentage (FECR %) was calculated utilizing the undermentioned formula:

$$\text{FECR \%} = [1 - (\frac{\text{mean EPG treatment}}{\text{mean EPG control}})] \times 100$$

Having said, the same aforementioned process was applied for detection of resistance against avermectin (two farms) and levamisole (two farms). The dewormers used in this survey were obtained from a local veterinary clinic. Moreover, RESO computer program (CSIRO Animal Health Research Laboratory, Private Bag 1, Parkville, Vic. 3052, Australia) was exploited to calculate the FECR data including arithmetic mean, variance of counts, FECR% and 95% confidence interval. According to Coles *et al.* (1992), resistance is rooted if (i) the FECR% is less than 95% (ii) the lower limit of 95% confidence interval is less than 90% (iii) If just one of the two norms is met, resistance is suspected. On the other hand, Gill (1996) has suggested that any negative values obtained from FECR% and lower limit confidence interval are equal to zero, interpreting that the resistance is extensively rampant and has reached the catastrophic level.

●Egg hatch assay (*in vitro* test)

This approach was conducted in accordance with the protocol that was adopted by World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P) to detect resistance against benzimidazole family members (Coles *et al.*, 1992). This *in vitro* technique was carried out for benzimidazole merely because other synthetic drugs are not ovicidal. On the other hand, egg hatch assay (EHA) provides an enhanced quantitative estimation of AR levels as compared to FECRT (Martin *et al.*, 1989). Faecal specimens from each farm (farms 1 and 2) were transported anaerobically to the laboratory to perform EHA. In brief, eggs were collected from pooled faecal samples on day 10-14 post-deworming, suspended

in distilled water and counted. Fenbendazole was dissolved in 0.3% Dimethylsulfoxide (DMSO) and mother solution was prepared as 50 µg ml⁻¹. The stock solution was serially diluted (0.0244-50 µg ml⁻¹ in a multiwell plate. The control well received only 1 ml solvent (0.3% DMSO). One ml (approximately 150 eggs ml⁻¹) of egg suspension was added to each well comprising the control well. Plate was incubated at 27°C ±1 for 48 hours and 70% relative humidity. After incubation, two drops of Lugol's iodine was added. At least 100 of the unhatched eggs (dead and embryonated) and hatched larvae were counted to calculate the hatching inhibition percentage (Coles *et al.*, 1992). The following formula was used for assessment of hatching inhibition (%):

$$\text{Hatching inhibition (\%)} = \frac{\text{P test}}{\text{P total}} \times 100$$

P test: number of unhatched or embryonated eggs.

P total: number of unhatched or embryonated eggs + Larvae (L₁).

LC₅₀ values were calculated for the eggs by probit analysis. Eggs with an LC₅₀ value in excess of 0.1 µg ml⁻¹ were reckoned as an indicative of antinematicidal (fenbendazole) resistance as suggested by Coles *et al.* (1992).

2.5. Statistical analysis

The diagnosis of resistance in deparasiting groups (after 10-14 days of deworming with fenbendazole, avermectin and levamisole) was scrutinized by RESO computer program after counting EPG of each animal in treatment and control groups. The level of resistance rampancy was assessed via estimation of FECR% and calculating lower limit of confidence interval using the above program.

The data collected from EHA for various concentrations of fenbendazole to evaluate its ovicidal activity against nematode eggs, one way ANOVA was applied utilizing Graph Pad Prizm (version 7). Tukey, as multiple comparison test, was utilized to compare among doses. All procured data were expressed as Mean±SE. For calculation of LC₅₀ (µg ml⁻¹) at 95% confidence interval for preventing 50% of egg hatching, probit analysis of LC₅₀ value on the EHA was applied.

3. Results

3.1. Involvement of gastrointestinal nematode species in infestations

The parasitic nematodes, naturally infecting goats, such as *Nematodirus*, *Trichuris* and *Marshallagia* larvae (L₃) were recognized post conduction of

qualitative, quantitative, coproculture and Baermann parasitological techniques (Soulsby, 1982; MAAF, 1986; Iqbal *et al.*, 2006) before and after deparasiting experimental goats in six farms (tables 1, 2 and 3).

3.2. Detection of fenbendazole resistance

3.2.1. Faecal egg count reduction test (*in vivo* test)

The data procured from EPG, FECRT, RESO Computer Program, after treatment of tentative animals with fenbendazole, had revealed that AR is rampant in the region. Details of the obtained data are exhibited in table 4.

3.2.2. Egg hatch assay (*in vitro* test)

The LC₅₀ of fenbendazole was estimated to be 2.11 µg ml⁻¹ (range 1.47-2.34), which was in excess to 0.1 µg ml⁻¹ suggesting emergence of

resistance among aforesaid GI nematodes (Coles *et al.*, 1992). Correlation between impacts of different concentrations of fenbendazole and hatching inhibition (%) through conducting EHA is shown in figure 1.

3.3. Detection of avermectin resistance

The data collected from EPG, FECRT, RESO Computer Program, after deworming of tentative animals with avermectin, had shown that AR was not prevalent in the area. Details of the procured data are displayed in table 5.

3.4. Detection of levamisole resistance

The data obtained from EPG, FECRT, RESO Computer Program, after treatment of tentative animals with levamisole, had revealed that AR was not spread in the study zone. Details of the collected data are demonstrated in table 6.

Table 1 Pre-deparasiting (0 day) and post-deparasiting (10-14 days) percentage of nematode larvae (L₃) in the experimental goats chosen for detection of resistance against fenbendazole based on pooled faecal specimens collected from farm 1 and 2

<u>Pre-deparasiting L₃ (%) of <i>Nematodirus</i>, <i>Trichuris</i> and <i>Marshallagia</i></u>			
Farms/groups	<i>Nematodirus</i> %	<i>Trichuris</i> %	<i>Marshallagia</i> %
<u>Farm 1 (Kandinawa district)</u>			
Fenbendazole group	39	31	30
Control group	36	27	37
<u>Farm 2 (Shamamic district)</u>			
Fenbendazole group	35	33	32
Control group	36	35	29
<u>Post-deparasiting L₃ (%) of <i>Nematodirus</i>, <i>Trichuris</i> and <i>Marshallagia</i></u>			
<u>Farm 1 (Kandinawa district)</u>			
Fenbendazole group	40	33	27
Control group	38	32	30
<u>Farm 2 (Shamamic district)</u>			
Fenbendazole group	34	35	31
Control group	35	33	32

Table 2 Pre-deparasiting (0 day) and post-deparasiting (10-14 days) percentage of nematode larvae (L₃) in the experimental goats chosen for detection of resistance against avermectin based on pooled faecal specimens collected from farm 3 and 4

<u>Pre-deparasiting L₃ (%) of <i>Nematodirus</i>, <i>Trichuris</i> and <i>Marshallagia</i></u>			
Farms/groups	<i>Nematodirus</i> %	<i>Trichuris</i> %	<i>Marshallagia</i> %
<u>Farm 3 (Kandinawa district)</u>			
Avermectin group	38	33	29
Control group	36	34	30
<u>Farm 4 (Shamamic district)</u>			
Avermectin group	37	35	28
Control group	35	33	32
<u>Post-deparasiting L₃ (%) of <i>Nematodirus</i>, <i>Trichuris</i> and <i>Marshallagia</i></u>			
<u>Farm 3 (Kandinawa district)</u>			
Avermectin group	slightly obtained	slightly obtained	slightly obtained
Control group	37	32	31
<u>Farm 4 (Shamamic district)</u>			
Avermectin group	slightly obtained	slightly obtained	slightly obtained
Control group	34	32	34

Table 3 Pre-deparasiting (0 day) and post-deparasiting (10-14 days) percentage of nematode larvae (L₃) in the experimental goats chosen for detection of resistance against levamisole based on pooled faecal specimens collected from farm 5 and 6

<u>Pre-deparasiting L₃ (%) of <i>Nematodirus</i>, <i>Trichuris</i> and <i>Marshallagia</i></u>			
Farms/groups	<i>Nematodirus</i> %	<i>Trichuris</i> %	<i>Marshallagia</i> %
<u>Farm 5 (Kandinawa district)</u>			
Levamisole group	36	34	30
Control group	39	35	26
<u>Farm 6 (Shamamic district)</u>			
Levamisole group	38	33	29
Control group	36	33	31
<u>Post-deparasiting L₃ (%) of <i>Nematodirus</i>, <i>Trichuris</i> and <i>Marshallagia</i></u>			
<u>Farm 5 (Kandinawa district)</u>			
Levamisole group	poorly obtained	poorly obtained	poorly obtained

Control group	40	32	28
<u>Farm 6 (Shamamic district)</u>			
Levamisole group	poorly obtained	poorly obtained	poorly obtained
Control group	35	34	31

Table 4 Status of resistance among *Nematodirus*, *Trichuris* and *Marshallagia* populations on day 10-14 post-deparasiting with fenbendazole based on RESO program

Farm/groups	Mean EPG ±SE	Confidence interval 95% Lower	FECR%	Rampancy of resistance
<u>Farm 1 (Kandinawa)</u>				
Fenbendazole group	204.27±19.95	63.00	70.72	Resistant
Control group	697.67±50.67	-	-	-
<u>Farm 2 (Shamamic)</u>				
Fenbendazole group	164.27±17.65	73.8	79.55	Resistant
Control group	803.07±58.33	-	-	-

EPG: Egg per gram of faeces

FECR%: Faecal egg count reduction percentage

Table 5 Status of resistance among *Nematodirus*, *Trichuris* and *Marshallagia* populations on day 10-14 post-deparasiting with avermectin based on RESO program

Farm/groups	Mean EPG ±SE	Confidence interval 95% Lower	FECR%	Rampancy of resistance
<u>Farm 3 (Kandinawa)</u>				
Avermectin group	46.67±5.05	92.91	93.92	Suspected
Control group	768.07±51.15	-	-	-
<u>Farm 4 (Shamamic)</u>				
Avermectin group	48.87±5.80	90.7	92.98	Suspected
Control group	696.07±45.34	-	-	-

EPG: Egg per gram of faeces

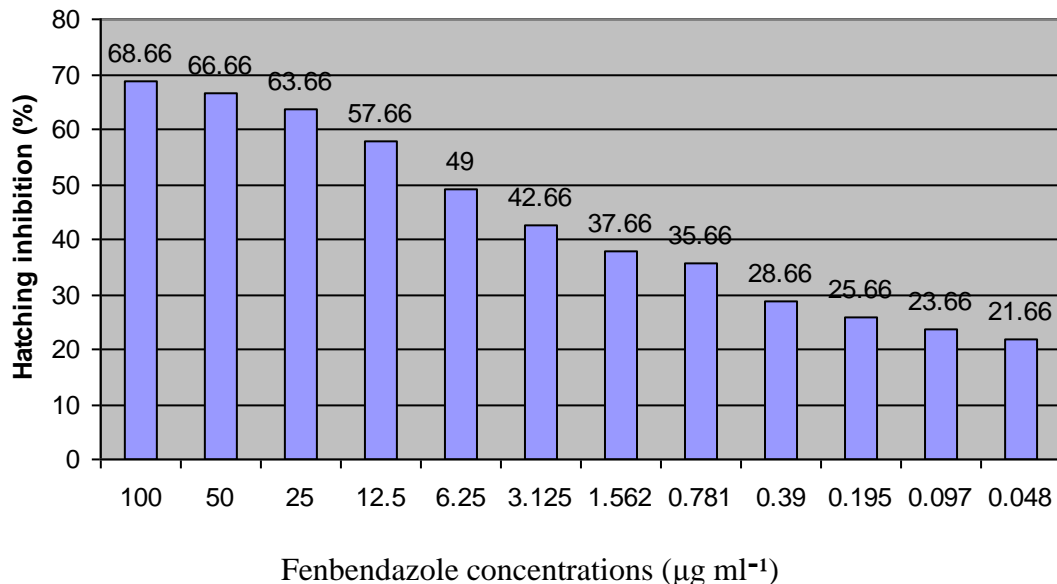
FECR%: Faecal egg count reduction percentage

Table 6 Status of resistance among *Nematodirus*, *Trichuris* and *Marshallagia* populations on day 10-14 post-deparasiting with levamisole based on RESO program

Farm/groups	Mean EPG ±SE	Confidence interval 95% Lower	FECR%	Rampancy of resistance
Farm 5 (Kandinawa)				
Levamisole group	26.33±2.21	95.1	95.99	Susceptible
Control group	656.80±38.00	-	-	-
Farm 6 (Shamamic)				
Levamisole group	25.40±1.51	95.7	96.38	Susceptible
Control group	702.47±41.24	-	-	-

EPG: Egg per gram of faeces

FECR%: Faecal egg count reduction percentage

**Figure 1** Correlation between the effects of different concentrations of fenbendazole and hatching inhibition (%)

4. Discussion

Undoubtedly, AR is counted a key bottleneck to control small ruminant GI nematodes and escalating concern for human nematodes owing to the wide use of antinematocidals, particularly in the under-developed nations (Geary, 2012; Furgasa *et al.*, 2018). Actually, this study is reckoned a first attempt to detect AR in goats in Kurdistan region; however, few surveys have been carried out to diagnose AR in sheep (Ahmed *et al.*, 2015; Ahmed *et al.*, 2019). After treatment of experimental goats in farms 1 and 2 with

fenbendazole, coprocultures produced sufficient individuals of nematodes L₃ (table 1). The FECR values in farms 1 and 2 were 70.72% and 79.55% respectively, while lower limit values of confidence intervals 95% in farms 1 and 2 were 63% and 73.8% respectively (table 4). Additionally, the LC₅₀ value was 2.11 µg ml⁻¹ (range 1.47-2.34) after performing EHA with fenbendazole (it has ovicidal activity as well) (figure 1). Definitely, the previous data are strong indications for the prevalence of resistance among GI roundworms (Coles *et al.*, 1992) of goats on

the trialed farms. In light of the current results, fenbendazole resistance is deemed multi-specific, which means emerging AR in one flock in numerous genera of parasitic roundworms including *Nematodirus*, *Trichuris* and *Marshallagia*. On the other hand, in tropical, sub-tropical and North American countries, fenbendazole resistance in small ruminants is often associated with *Haemonchus contortus* (Falzon *et al.*, 2013; Hamad *et al.*, 2014; Kalkal *et al.*, 2019). It is imperative to draw attention to some possible factors responsible in developing fenbendazole resistance in under-developed territories including our region which comprise insufficient dose, frequent annual use, bad quality of the dewormer, storage conditions, deparasiting animals with the same drug 3-4 times annually, and improper drenching procedure (Hoekstra *et al.*, 1997; Afaq, 2003; Saeed, 2007; Furgasa *et al.*, 2018). Probably, among the aforementioned factors, recurrent yearly employment of the same drug, which causes selective pressure on the parasite, conduces to occurrence of mutation (loss of dewormer binding) at β -tubulin isotype 1 (dewormer target) in parasitic nematodes (Beech and Silvestre, 2010). Moreover, according to the veterinarian files in the study sites and experience of the first author (Veterinary clinician since last century), usually local Kurdish shepherds prefer to drench their livestock with fenbendazole and its counterparts of the benzimidazole family recurrently as compared to other dewormer families. Consequently, this practice has led to emergence and rampancy of resistance (Hamad, 2012) in the region.

Avermectin and its counterparts were commercialized in 1980 and introduced to the therapy recipe in Kurdistan region slowly in 1991, so for many years, local farmers have not been adapted to use it because this drug was new for them at that time. On the other hand, the route of administration of this dewormer is injection subcutaneously, so livestock raisers try to avoid it (local veterinarian file). Hence, use of avermectin is less than fenbendazole and its counterparts. Post-therapy of goats with avermectin in farms 3 and 4, coprocultures yielded slight numbers of nematodes L₃ (table 2). Furthermore, the FECR values in farms 3 and 4 were 93.92% and 92.98% respectively, whilst the lower limit values of confidence intervals 95% in farms 3 and 4 were

92.91% and 92.7% respectively (table 5). Consequently, and according to Coles *et al.* (1992), the resistance is suspected against avermectin in farms 3 and 4.

Levamisole, as a cholinergic agonist working at nicotinic acetylcholine receptors on the surface of the roundworm muscle cells and at the neuromuscular junction, has no antitrematocidal, anticestocidal and ovicidal activity (Taylor *et al.*, 2007). In most areas of the world including Kurdistan region, levamisole is being used against the lung worm, *Dictyocaulus filaria* of small ruminants and it has a low safe margin (Radostits *et al.*, 2007; Besier *et al.*, 2016). Thus, its employment by livestock raisers is too rare as compared to fenbendazole and its counterparts. After drenching the remedial dose of levamisole to tentative goats in farms 5 and 6, coprocultures produced poor numbers of nematodes L₃ (table 3). Besides, the FECR values in farms 5 and 6 were 95.99% and 96.38% respectively, whilst the lower limit values of confidence intervals 95% in farms 5 and 6 were 95.1% and 95.7% respectively (table 6). Accordingly, and pursuant to Coles *et al.* (1992), the parasitic worms were susceptible and no resistance was detected against levamisole in farms 5 and 6. In this regard, Coles *et al.* (1995) have mentioned that the resistance will develop if annual drench exceeded two times. Having said, the usual annual use of this dewormer is only two times, one in autumn and other one in spring (local veterinarian record).

5. Conclusions

In the light of results of the present original study pertaining Kurdish goat breeds in our region, it has been concluded that prevailing GI nematodes; *Nematodirus*, *Trichuris* and *Marshallagia* are resistant to fenbendazole, whilst the resistance is suspected to avermectin. On the other hand, the abovesaid parasites are susceptible to levamisole. Farmers in the study area often prefer fenbendazole and its counterparts in drenching their livestock on other drugs such as avermectin and levamisole which belong to different families. Having said, the livestock raisers drench goats (the amount of drugs should be double as compared to sheep) the dose rate of sheep, which in turn, could lead to emergence of resistance especially against fenbendazole and its counterparts.

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Conflict of interest

The authors of this research article attest that there is no any conflict of interest with other authors concerning this manuscript.

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