

# First report of multiple drug-resistant gastrointestinal nematodes of sheep in Arbat District, Sulaymaniyah, Iraq detected by *in vivo* and *in vitro* methods

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## Abstract

**Introduction:** The control of gastrointestinal nematodes (GINs) in small ruminants is principally dependent on anthelmintic therapy, which encounters the rising problem of anthelmintic resistance (AR) development. Veterinarians reported anthelmintic failure in several sheep farms in Arbat District, Sulaymaniyah, northern Iraq, which called for a systematic study about the efficacy of three commonly used drugs: albendazole, ivermectin, and levamisole. **Material and Methods:** A faecal egg count reduction test (FECRT) was conducted to elucidate the anthelmintics' efficacies, with coprocultures to determine the GINs parasitising sheep in the study area. Larval development assays (LDAs) were conducted to determine the drugs' median inhibitory concentrations (IC<sub>50</sub>). **Results:** The FECRT revealed that AR was widespread to all three drugs, and ivermectin was the least effective, reducing the faecal egg counts (FECs) by 50.5% to 57.1%. The coprocultures revealed that the GIN genera of sheep in Arbat District were *Trichostrongylus*, *Nematodirus*, and *Trichuris*, and the resistance was mainly due to *Trichostrongylus* species. The mean IC<sub>50</sub> of albendazole, ivermectin, and levamisole were 0.073 ng/mL, 7.97 ng/mL, and 1.43 ng/mL, respectively. **Conclusion:** This study is the first Iraqi report of AR confirmed by both *in vivo* FECRT and *in vitro* LDA methods.

**Keywords:** albendazole, anthelmintic resistance, ivermectin, levamisole, *Trichostrongylus*, larval development assay.

## Introduction

The Nematoda phylum includes many helminth species parasitising humans and domestic and wild animals (23). Gastrointestinal nematodes (GIN) are globally distributed in small ruminants and negatively impact animal health and production. All grazing sheep are exposed to GIN infections, which are regarded as one of the most important ovine diseases worldwide and cause weight loss, diarrhoea, and death (7). Production losses result from the loss of appetite, tissue and organ damage, reduced feed conversion, and deaths of heavily infected animals. The amounts of weight gain and milk production in infected animals are 15% and 22% less than those of noninfected animals, respectively, and in addition, the cost of anthelmintic therapies indirectly impacts livestock productivity (19).

Different strategies are implemented for GIN control in domestic animals, but anthelmintic therapy is the most widespread method of helminth control globally (9). The most commonly used anthelmintics

are benzimidazoles, macrocyclic lactones, or imidazothiazoles (1). These are broad-spectrum drugs with wide safety margins, but their extensive use has led to anthelmintic resistance (AR) development, which occurs when a drug's recommended dosage cannot effectively treat the infected animal (8). The arising and spread of AR are accelerated by underdosing, repeated use of the same anthelmintic, and treatment of the whole flock (3).

Parasitic diseases and the emergence of AR are significant human health problems (25). This resistance is widespread in sheep-producing countries, is a persistent and growing issue in all parts of the world, and requires immediate attention (11). Several reports of AR development have been published in recent years, and it has become a global problem in sheep and goats in the last three decades (2, 22).

Successful helminth control strategies partially depend on accurately detecting AR in an area. However, several factors other than resistance may be responsible for anthelmintic therapy failure. For example, faulty

flock drenching equipment may lead to livestock not being thoroughly dewormed. Anthelmintic underdosing due to inaccurate estimation of the animal's weight or anthelmintic dose is another common cause of anthelmintic failure (24).

Various *in vitro* and *in vivo* AR detection methods in GINs have been developed, but most of these tests are not adequately reliable, reproducible, or sensitive (24). The faecal egg count reduction test (FECRT) is the most commonly used method to estimate anthelmintic efficacy *in vivo* by comparing pre-treatment and post-treatment faecal egg counts (FECs) (4). Larval development assays (LDAs) are also considered reliable methods for investigating the progress of AR to different anthelmintic drugs, including albendazole, ivermectin, and levamisole (12).

One of the drawbacks of the FECRT is that it does not provide accurate information about the nematode species parasitising the tested animals and endowed with resistance. Some nematodes such as *Teladorsagia colubriformis*, *Nematodirus* spp., and *Ostertagia circumcincta* produce fewer eggs than other species such as *Haemonchus contortus* (24), and therefore species knowledge is important for sound interpretation of FECRT results. Hence, larval culture is used to determine the nematode species responsible for AR development.

In the Kurdistan Region, northern Iraq, the sheep population numbers more than three million head and is mainly raised on pasture grazing. Their farmers use anthelmintics to reduce the burden of GIN infection. The most commonly used dewormers are albendazole, ivermectin, and levamisole, administered *via* drenching. Recently, incidents of the common anthelmintics' failure were reported by veterinary practitioners on several farms in the Arbat District, one of the Sulaymaniyah districts in the Kurdistan Region of Iraq. Accordingly, this study investigated the occurrence of resistant sheep GINs on three farms in Arbat using *in vivo* FECRT, larval culture, and *in vitro* LDA.

## Material and Methods

### Farm selection and anthelmintic treatment.

Anthelmintics are commonly used to control soil-transmitted nematodes in small ruminants in the north of Iraq. This study was conducted to investigate AR occurrence in Arbat District, northeastern Iraq, located 35.28°–35.38°N and 45.13°–45.29°E. Veterinary practitioners have reported the failure of anthelmintic therapy on approximately ten sheep farms in the region.

Arbat District is located 23 km southeast of the Sulaymaniyah Governorate, Kurdistan Region, northern Iraq. The district comprises 71 villages, and mainly sheep and cattle farming represents the primary income source. The sheep population is around 65,000, and the animals are kept in different size flocks and raised on free pasture throughout the year.

Farmers in Arbat District drench their sheep with anthelmintics once or twice a year to control GIN infections. Avermectins are the most commonly used, followed by imidazothiazoles and benzimidazoles. Veterinary practitioners reported the therapeutic inefficiency of antinematodal drugs in several herds after drenching the sheep with several anthelmintics. These incidences urged the carrying out of a standardised study to investigate the efficacy of the commonly used anthelmintics in this region.

Ten farms were visited to investigate their management practices. A questionnaire was distributed among the farmers, putting several questions about the number, age, and sex of the sheep on each farm, the date of previous treatment, and the type of anthelmintic used. Farms were included in the study by meeting qualifying criteria: keeping  $\geq 40$  sheep between the ages of six and eighteen months, grazing them on pasture, not having treated them with anthelmintics for the previous three months, and scoring a mean pre-treatment faecal egg count of  $\geq 200$  eggs per gram (EPG) for their animals.

Three farms met the selection criteria and were included in this study. The combined number of sheep raised on the studied farms was 3,623, accounting for about 5.6% of Arbat's sheep population. The other farms were excluded for various reasons, such as carrying out treatment with anthelmintics in the previous 12 weeks, keeping too few animals of the required age, or the owners' declining to participate in the study.

Forty sheep of both sexes, 6 to 18 months old, were selected from each farm, divided into four groups of 10, and tagged appropriately. The first group served as the control and was drenched with distilled water. Groups 2 to 4 were administered a single oral dose of albendazole, ivermectin, or levamisole, respectively.

The recommended anthelmintic dosages of 5.0 mg/kg of albendazole (Albenol-100), 0.2 mg/kg of ivermectin (Intermectin Drench), and 10.0 mg/kg of levamisole (Leva-200) were used. All drugs were produced by Interchemie (Venray, the Netherlands). Each animal's dose was calculated according to its weight and administered using an automatic drenching gun. The study protocol was submitted to and approved by the Animal Care and Use Committee (ACUC) at the College of Veterinary Medicine, University of Sulaimani.

### Sample collection and faecal egg count reduction

**test.** Faecal samples were collected twice from each sheep. The first sample was collected just before the anthelmintic administration, and the second was taken 14 days post-treatment. Faecal pellets (5–15 g) were taken directly from each animal's rectum, put in suitable containers, labelled, and stored in an icebox. The samples were brought to the Research Laboratory at the College of Veterinary Medicine, University of Sulaimani, and kept refrigerated overnight. An FEC was conducted for each sample applying a modified McMaster method. The procedure started with weighing 3.0 g of faeces into a container and adding 42.0 mL

of water. The mixture was then homogenised using a laboratory stirrer and shaken until all pellets were broken up. The homogenised mixture was poured into a bowl through a 0.15 mm aperture, 20.0 cm diameter sieve. Fifteen millilitres of the filtrate were put into a centrifuge tube, and this was centrifuged for two minutes at 1,500 rpm. The supernatant was gently discarded, the tube was agitated to loosen the sediment, and a saturated sodium chloride solution was added to give the same volume (15.0 mL) as before. The tube was shaken by inverting five or six times, and immediately a sample was withdrawn with a pipette. The two chambers of a McMaster slide were filled with the withdrawn mixture. Nematode eggs were observed under an inverted microscope with 40× magnification power. All the nematode eggs under the two ruled grids (total volume of 0.3 mL) were counted, and the final number was multiplied by 50 to give the EPG in the faecal sample (9).

The FECRT determines an anthelmintic's efficacy *in vivo* by comparing the post-treatment FEC to the pre-treatment FEC and calculating the faecal egg count reduction (FECR). This was calculated as

$$100 \times \left(1 - \frac{\text{post-treatment FEC in the treatment group}}{\text{post-treatment FEC in the control group}}\right)$$

and the 95% confidence interval as  $\text{FECR} \pm 1.96\sqrt{\text{variance}}$ .

Faecal egg count reduction percentages below 95% and/or lower 95% CI limits equal to or lower than 90% indicated resistance. If only one condition were met, resistance was suspected, and the herd was considered resistant if the FECR was < 95% and the lower CI was < 90% (4, 10).

The efficacies of albendazole, ivermectin and levamisole in the study region were compared statistically using a one-way analysis of variance (ANOVA), followed by Duncan's *post hoc* test. The Statistical Package for the Social Sciences (SPSS version 24.0) program from IBM (Armonk, NY, USA) was used to conduct the statistical analysis.

**Coprocultures.** Faecal samples were collected before and after anthelmintic treatment, and 3.0 g of faecal pellets from each sheep on the same farm and in the same treatment group were mixed in a glass jar. Distilled water was added to the jar to bring the consistency to a suitable level, and the jar was incubated at 25°C for 7 d for third-stage nematode larval development. The pre-treatment samples were cultured to determine the GINs parasitising sheep in the study area. The post-treatment faecal samples were cultured to determine the genera that survived the anthelmintic therapy and acquired resistance.

After incubation for one week, the third-stage larvae (L3s) were collected by the larvoscopic Baermann technique, previously described by Coles *et al.* (4). The harvested larvae were put on a glass slide, stained with Lugol's iodine and covered with a coverslip. The L3s were examined under an inverted microscope (100 × magnification). The first 100 larvae detected in the slide were morphologically identified to the genus level (26), the proportion of each genus in the first

100 L3s was calculated, and the reduction percentage of each nematode genus was determined using the following formula:

$$\text{Reduction (\%)} = 100 \times \left(1 - \left(\frac{\text{mean genus FEC post-treatment}}{\text{mean genus FEC pre-treatment}} \times \frac{\text{mean genus FEC pre-control}}{\text{mean genus FEC post-control}}\right)\right).$$

**Larval development assay.** Larval development assays (LDAs) were performed to assess the drugs' effect on the GIN eggs' hatchability and calculate their median inhibitory concentration (IC<sub>50</sub>) *in vitro*. The samples used for the LDAs were from animals not treated with an anthelmintic for three months. The method described by Coles *et al.* (5) was implemented.

Tests were performed in 96-well microtitre plates. Nematode eggs were recovered from the faeces by passage through sieves with 250 µm and 75 µm aperture sizes, followed by centrifugation on a two-step sucrose gradient (10% and 25% sucrose). The eggs were recovered from the top layer of the 25% sucrose and washed over a 25 µm sieve with water to remove residual sucrose.

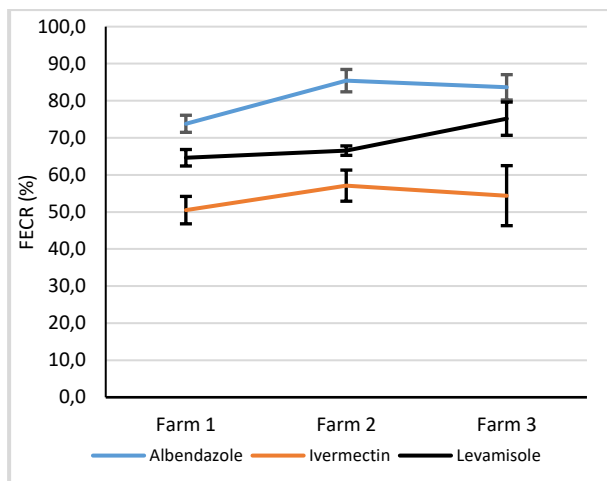
The eggs were agitated gently in an 8.4 mg/L sodium hypochlorite solution for 12 min and subsequently washed with a large volume of water. The eggs had distilled water added in order that after the addition of amphotericin B (at a final concentration of 25.0 mg/mL) and tylosin tartare (at a final concentration of 800 µg/mL), 30 µL contained 50–60 eggs, and the solution was used immediately for larval development assays (6). Assay plates were prepared by adding 150 µL of nutrient broth to the wells of a 96-well plate. Egg solution in a 30 µL volume was added to each well.

The microtitre plates were put in a humid chamber to prevent drying and incubated overnight at 26°C. The next day, 2.0 µL of a growth medium was added to each well (14). The medium constituents were Earle's salt solution (10% v/v), yeast extract (1% w/v), sodium bicarbonate (1 mM), and saline solution (0.9% sodium chloride w/v). The LDA used was a modified version of the method described initially by Taylor *et al.* (24). In the modified method, the culture medium comprised lyophilised *Escherichia coli* and 2.0 µL of a solution of *E. coli* cells in lysogeny broth (LB) and 6.0 µL of water. The final volume of the egg solution was adjusted to give 300 eggs/mL. Three concentrations of albendazole (0.1 µg/mL, 0.3 µg/mL, and 0.9 µg/mL), levamisole (1.0 µg/mL, 3.0 µg/mL and 9.0 µg/mL) and ivermectin (3.5 ng/mL, 10.5 ng/mL and 31.5 ng/mL) were tested.

The plates were incubated at 26°C for additional five days. After that, 10 µL of Lugol's iodine solution was added to each well, and the content was put on a glass slide. The number of fully developed infective-stage larvae (L3) present in each well was counted. The percentage of L3 in the treatment wells was calculated by the formula  $100 \times (\text{number of L3 in T} / \text{number of L3 in C})$ , where T and C are the wells containing the test drugs and control wells, respectively. The IC<sub>50</sub> of each anthelmintic was calculated using the Microsoft Excel 2016 program, and all tests were run in triplicate.

## Results

**Determination of the tested drugs' efficacies *in vivo*.** Gastrointestinal nematodes resistant to albendazole, ivermectin, and levamisole were present on the three farms. All the tested anthelmintics proved ineffective in reducing the post-treatment FEC by 95% (Fig. 1). However, the FECR by albendazole was significantly higher than those achieved by the other anthelmintics, and levamisole was more efficacious than ivermectin ( $P < 0.05$ ). Ivermectin reduced the FECR by 50.5%, 57.1%, and 54.4% on the three farms.



**Fig. 1.** Faecal egg count reduction (FECR) by albendazole, ivermectin, and levamisole on three farms in Arbat District, Sulaymaniyah, Iraq. All anthelmintics failed to reduce the post-treatment FEC by 95%. Values represent the average FEC in 10 sheep (lines) and 95% confidence intervals (error bars)

### Gastrointestinal nematodes in the study area.

The FEC in the pre-treatment samples ranged between 604.2 and 764.1 EPG. The larval culture revealed that *Trichostrongylus* spp. were the most common L3s (76%–86%), while *Nematodirus* spp. and *Trichuris* spp.

were also detected (Table 1). *Trichostrongylus* species reduction did not reach 95% following treatment with any of the three drugs. The nematodes of this genus were reduced by 63.3%, 66.2%, and 70.5% on the studied farms following levamisole treatment (Fig. 2). *Trichostrongylus* reduction ranged between 73.1% and 82.4% after albendazole treatment and between 49.3 and 52.4% after ivermectin treatment. These results indicated that the resistance is primarily caused by resistant *Trichostrongylus* species since they were the most prevalent in the pre-treatment samples.

Larval cultures of the post-treatment faecal samples revealed that *Nematodirus* was reduced by 50% in farm number 3 following albendazole treatment, indicating the emergence of albendazole-resistant *Nematodirus*. However, the genus was eliminated completely on the other farms.

The ivermectin-resistant GINs were *Trichostrongylus* spp. and *Nematodirus* spp. (Fig. 2), while *Trichuris* spp. were 100% susceptible on all three farms. *Trichuris* spp. were susceptible to all three drugs, except on farm number 3, where levamisole did not have this 100% effectiveness. The larval culture and FECR results revealed that AR was present in the Arbat District, primarily as a characteristic of *Trichostrongylus* and, to a lesser extent, *Nematodirus* species.

**Larval development assay.** The LDA was used to confirm the FECRT results and determine the *in vitro* IC<sub>50</sub> of albendazole, ivermectin, and levamisole. The results are shown in Table 2. The LDA results revealed that 0.3 µg/mL of albendazole completely inhibited nematode larval development *in vitro* on all three farms. The median inhibitory concentrations for albendazole on the three farms were 0.076 ± 0.004 µg/mL, 0.071 ± 0.004 µg/mL, and 0.072 ± 0.007 µg/mL, respectively. This outcome indicated that albendazole effectively inhibited larval nematode development at the recommended concentrations.

**Table 1.** Percentage of third-stage larvae in the first 100 larvae isolated from a pooled post-treatment faecal sample of the control and treated groups

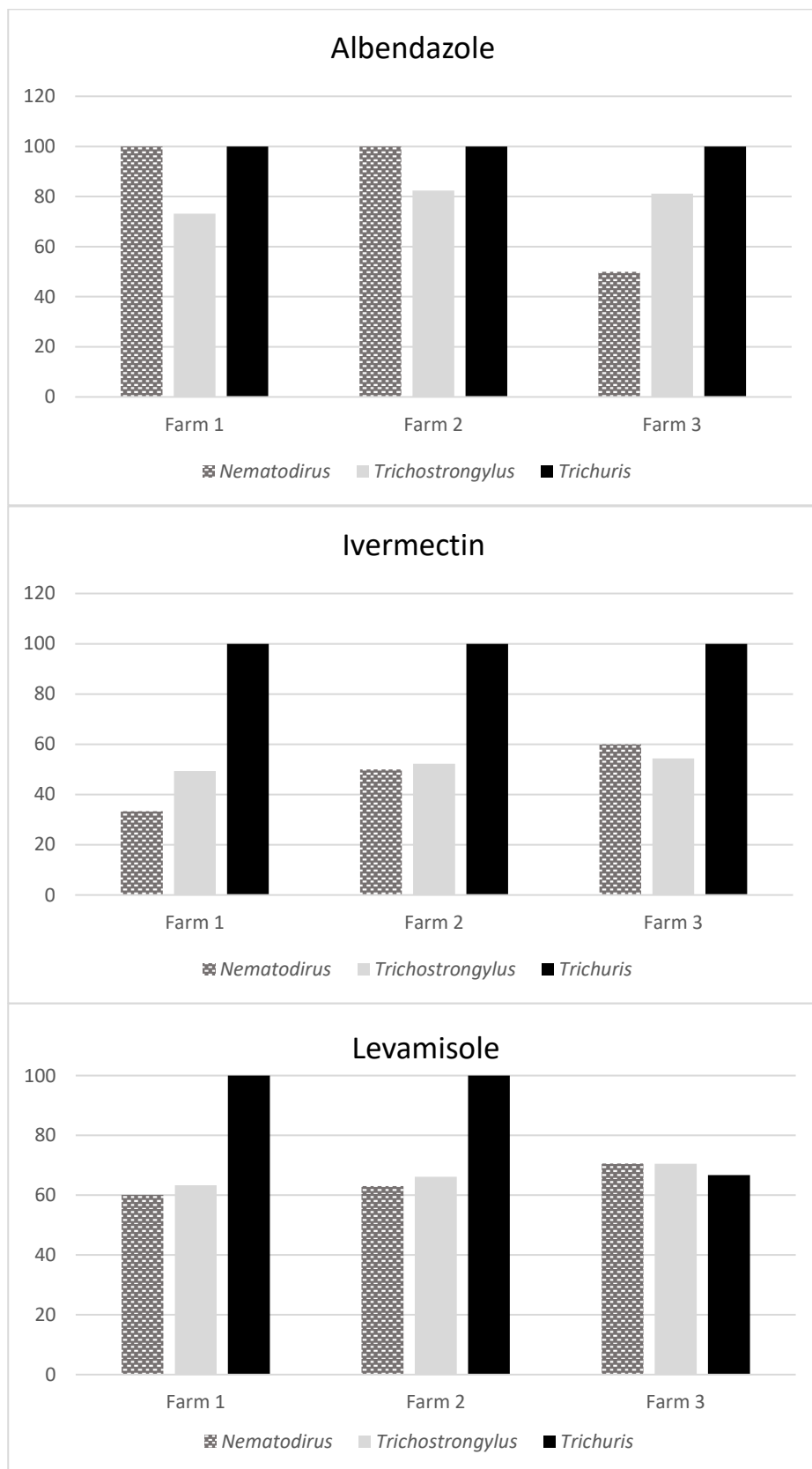
Farm	Pre-treatment FEC* (eggs/g)	Percentage of larvae by genus		
		<i>Trichostrongylus</i>	<i>Nematodirus</i>	<i>Trichuris</i>
1	764.1 ± 45.2	82.0	12.0	6.0
2	760.0 ± 48.1	76.0	15.0	9.0
3	604.2 ± 37.8	86.0	8.0	6.0

\* Values represent mean pre-treatment FECs in 40 sheep ± SEM

**Table 2.** Median inhibitory concentration (IC<sub>50</sub>) of anthelmintics preventive of nematode egg hatchability

Farm	IC <sub>50</sub>		
	Albendazole (µg/mL)	Ivermectin (ng/mL)	Levamisole (µg/mL)
1	0.076 ± 0.004	8.030 ± 0.692	1.470 ± 0.090
2	0.071 ± 0.004	7.670 ± 0.622	1.450 ± 0.140
3	0.072 ± 0.007	8.200 ± 0.615	1.390 ± 0.120

Values represent the mean of three tests ± SEM



**Fig. 2.** Percentage of reduction in *Nematodirus* spp., *Trichostrongylus* spp., and *Trichuris* spp. after treatment with anthelmintics on Arbat's three sheep farms

Levamisole was also effective in inhibiting larval development, as a 3.0 µg/mL concentration inhibited approximately 82% of the larvae in all three farms. The IC<sub>50</sub> of levamisole were 1.47 ± 0.09 µg/mL, 1.45 ± 0.14 µg/mL, and 1.39 ± 0.12 µg/mL for the three farms. These results showed that the nematode larvae were susceptible to levamisole's inhibitory effects at the standard concentrations.

Ivermectin did not effectively inhibit larval developments at the recommended concentration of 3.5 ng/mL, and only at a concentration as high as 31.5 ng/mL did it inhibit larvae, doing so for about 79%. Ivermectin's IC<sub>50</sub> values were 8.03 ± 0.69 ng/mL, 7.67 ± 0.62 ng/mL, and 8.20 ± 0.62 ng/mL, respectively.

The LDA results showed that albendazole was the most effective anthelmintic drug at inhibiting nematode larval development at the prescribed concentration. Levamisole inhibited larval progress to L3 at concentrations slightly higher than the recommendations. Ivermectin, in contrast, was ineffective in inhibiting larvae at the recommended concentration, and the IC<sub>50</sub> was about 2.3 times as high as that value.

## Discussion

Anthelmintic resistance is an increasing problem of small ruminant production worldwide that may render currently available drugs inefficient in the future, and accordingly, the development of control strategies has become a necessity (13). Implementing a suitable AR control program starts with identifying the common GINs and determining the extent of resistance in an area (15). In the north of Iraq, where the livestock industry is the key agricultural sector, GIN control is highly important, and investigating how AR is distributed among GIN species is required to improve the control strategy.

In the study area, GIN control is primarily dependent on anthelmintic treatment. This study investigated AR in Arbat District after reports of anthelmintic failure on several farms in the area. FECRT, considered the most reliable method of testing an anthelmintic drug's utility under field conditions (18), was used to elucidate the efficacy of albendazole, ivermectin, and levamisole *in vivo*. However, the method cannot identify the specific GINs in a given study area, and therefore larval cultures were conducted concomitantly to determine the GINs parasitising Arbat District sheep. The *in vitro* LDA was also used to confirm the FECRT results. The findings of this study confirmed the occurrence of AR to all the tested drugs, as none of them was able to reduce FECs by 95%. The larval cultures revealed that *Trichostrongylus* spp., *Nematodirus* spp., and *Trichuris* spp. were the common GINs in the study area. Also, it was shown that the reported resistance is due to *Trichostrongylus* and to a lesser extent *Nematodirus* spp.

The FECRT showed that ivermectin was the least efficacious anthelmintic in this study, while albendazole was the most effective. The questionnaire answers revealed that the drugs most commonly used by farmers in the study area were ivermectin, levamisole and albendazole, in order of frequency of implementation. Frequent use of an anthelmintic over an extended period usually provokes AR development (21). Ivermectin is an endectocide often used to control external arthropods and internal nematode parasites, which may justify its frequent use by sheep farmers. The drug's frequent use might be the reason why it was the least effective anthelmintic.

One of the FECRT disadvantages is that the results' accuracy is affected by such factors as underdosing. The tested drugs were administered to each animal to overcome this problem using recommended dosage rates based on each sheep's weight. Therefore, the AR reported by the FECRT cannot be a result of deficient dose administration and must only be due to the emergence of resistant GINs. Furthermore, the FECRT results were confirmed using the *in vitro* LDA.

The LDA was conducted to determine the drugs' IC<sub>50</sub> values, and the results indicated that AR against ivermectin had developed on all three farms. The drug's IC<sub>50</sub> ranged between 7.67 and 8.20 ng/mL, while previous studies reported IC<sub>50</sub> of 0.23 ng/mL (16, 20), meaning that ivermectin was only 3% as potent in the present study. In a previous study, the IC<sub>50</sub> of levamisole on *Trichuris muris* larvae was 1.75 µg/mL (27), close to the 1.39 µg/mL reported in this study. This result opposed the *in vivo* FECRT, as AR to levamisole was evident in the latter. Albendazole showed a potent effect in the LDA, as the drug's IC<sub>50</sub> of 0.071–0.076 µg/mL was in the range of recommended concentrations (10). This outcome also contrasted with the FECRT results. Many factors may lead to a false finding of decreased efficacy, such as defective equipment, wrong dosage, missed animals, inferior product, and mislabelled samples (17). However, these factors might not cause the discrepancy between these LDA results and those of the FECRT, as care was taken to avoid misconduct in executing the procedures. The daily egg production differs in different nematode species, affecting anthelmintic efficacy during FECRT (24), and because the effect of this variable was not eliminated in the study, it may explain why the LDA and FECRT results are at variance.

In conclusion, this study is the first report which reveals AR occurrence in sheep in Arbat District, northern Iraq, confirmed by FECRT and LDA. Resistance to ivermectin was evident and was principally due to the emergence of resistant *Trichostrongylus* species. The common GIN genera in the study area were *Trichostrongylus*, *Nematodirus*, and *Trichuris*. The results establish the ground for designing and executing an appropriate GIN control program in Arbat and other areas in the north of Iraq.

**Conflict of Interest Statement:** The authors declare that there is no conflict of interests regarding the publication of this article.

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**Animal Rights Statement:** All procedures that include animals were approved by the Animal Care and Use Committee at the University of Sulaimani under approval number AUP-2020-09.

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