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Evaluation of drug resistance to albendazole and levamisole against lung worms in goat flocks based on fecal larvae count reduction test

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Abstract

The over-use of anti-parasitic compounds as a method of control has led to insufficient effectiveness and widespread drug resistance worldwide. The aim of this study was to investigate the efficacy of albendazole and levamisole as anti-parasitic agents in a lung worm control program in goat flocks. During 2021 and 2022, a total of 110 goats (age of four months and above) were randomly selected from 11 herds in the north-western region of Iran including Saanen breed (both sexes of the same age). The results indicated that 3.60, 50.80 and 41.90% were respectively infected with *Dictyocaulus filaria*, *Muellerius capillaris* and *Proto-strongylus rufescens*, and generally all the lung parasites in goats of this region were resistant to albendazole and levamisole. Due to clinical importance of *D. filaria* in goats, the molecular analysis of two samples was also done. Sequencing results showed that the identified parasites were 100% similar to the reference sequences registered in the GenBank®. The results of this research showed low level of these anthelmintics efficacy against *Dictyocaulus* and *Muellerius*. Generally, the lung parasites in goats of this region are resistant to albendazole and levamisole. The *P. rufescens* showed high resistance to these drugs. Totally, it can be concluded that the level of drug resistance varies in different parts of the world; but, the frequencies of drug resistance in different parts of the world are not the same, requiring more studies.

Introduction

Parasitic lung diseases pose a significant challenge to the small ruminant industry, resulting in substantial economic losses.[1] Within the group of parasites being responsible for causing bronchitis in goats, *Dictyocaulus filaria* is the most pathogenic, *Muellerius capillaris* is the most prevalent but least pathogenic, and *Protostrongylus rufescens* and *Cystocaulus ocreatus* have intermediate levels of pathogenicity. The life cycle of *D. filaria* is direct; whereas, other lung worms need a snail or slug as an intermediate host.[2] The *D. filaria* transmission takes place during the cooler months. Conversely, *M. capillaris*, *P. rufescens* and *C. oceratus* are transmitted when snails or slugs are present, typically occurring in spring or summer. Infected snails and slugs can carry over transmission from one year to the next if they survive in winter.[3] In *Dictyocaulus* species, first-stage larvae (L1) are shed in faces, where they develop into infective third-stage larvae (L3) in the environment. The L3 are ingested by ruminants while grazing and migrate to the respiratory system, where they mature into adults within the lumen of bronchi and bronchioles. Females shed eggs, being embryonated in the lungs and hatched within the gastrointestinal tract, before being shed in feces. Among the Metastrongyloidea, *M. capillaris*, *P. rufescens* and *C. ocreatus* have indirect life cycles, requiring gastropod intermediate hosts for the development of L1 to the infective L3.[4] Adult females lay eggs in lungs and L1 hatch within the airways and pass, before invading susceptible terrestrial snail hosts. Small ruminants become infected by inadvertently ingesting snails harboring infective L3.[5] Ingested L3 migrate to the respiratory system, maturing to todioecious adults. Environmental conditions may directly influence the occurrence of lung worm infection in different host species. Also, it should be noted that climate change, loss of biodiversity, animal trade and lack of large-scale surveillance can play key roles in fluctuations observed in the prevalence and diversity of species in different regions.[6,7]

Anthelmintic resistance refers to the reduction in effectiveness of a particular anthelmintic drug against a

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group of parasites that would normally be susceptible to it.[8] The indiscriminate and incorrect use of these drugs is exacerbating the problem. In particular, small ruminant nematodes are experiencing a more severe drug resistance issue, requiring fundamental changes in control methods.[9] Iranian studies have shown a high prevalence of broad spectrum anthelmintic resistance in gastrointestinal helminths of ruminants.[10]

Numerous techniques exist for detecting resistance to anti-parasitic drugs. In many regions, nematode drug resistance is assessed through *in vivo* methods, which involve measuring the decrease in the number of eggs in feces before and after treatment, examining the hatching of larvae, observing treated animals through necropsy, and evaluating the growth of larvae.[11-14]

In Iran, livestock farmers frequently administer benzimidazoles, specifically albendazole, to their animals irrespective of the presence of parasitic infection. The use of this anti-parasitic compound has been in practice since 1960s as a strategic plan to combat worm infections in small ruminants. However, albendazole has been used for different purposes alongside the strategic treatment.[15]

There is limited information available regarding the potential presence of drug-resistant nematodes in Iran. However, resistance to albendazole was reported in gastrointestinal nematodes of sheep in Khozestan province of Iran and in another study, gastrointestinal nematodes resistances to albendazole and fenbendazole were reported in Iran.[15-17]

This study aimed to evaluate the efficacy of two very common drugs in the local market, *i.e.*, albendazole and levamisole, by fecal larvae count reduction test (FLCRT) in lung worm-positive goats in northwest of Iran. Due to the clinical importance of *D. filaria* in goats, the molecular analysis of samples was also done.

Materials and Methods

Collection. During 2021 and 2022, a total of 110 goats (age of four months and above) were randomly selected from 11 herds in the north-western region of Iran including Saanen breed (both sexes of the same age; Fig. 1). The goats were allowed to graze freely during the day and kept in the pastures at nights. All goats were in good health conditions based on clinical examinations during the time of sampling and had not been treated with anthelminthic drugs at least eight weeks prior to sampling. The collection of fecal samples was carried out in two stages including before treatment and 14 days after treatment.

At least 10.00 g fecal specimens were directly collected from each animal, carefully placed in plastic bags and then, transferred under the cold chain to the Laboratory of Parasitology at the Faculty of Veterinary Medicine in Urmia University, Urmia, Iran.

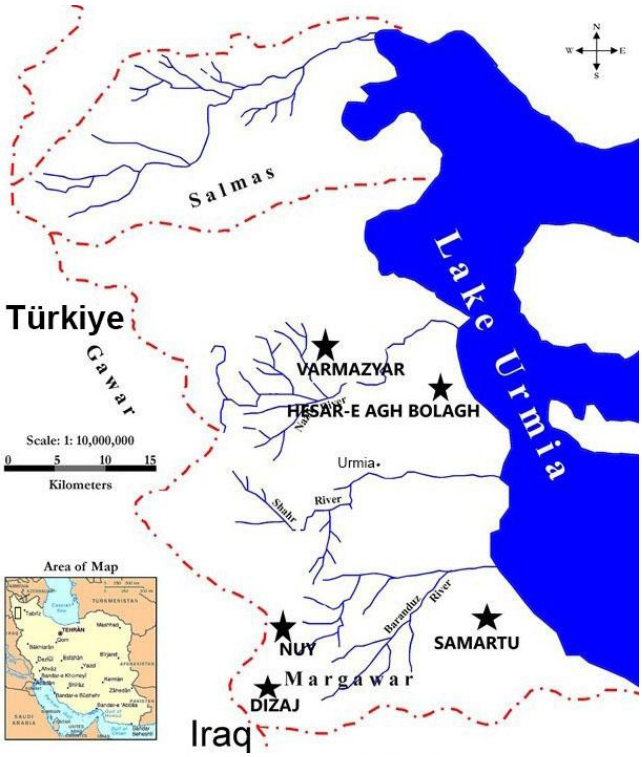

Fig. 1. Geographical location of the sampled villages in the north west of Iran. Asterisks indicate the areas of sampling.

Baermann technique. To determine the larval count *per* g of feces (LPG), the Baermann technique was utilized using standard Baermann apparatus.[18] The apparatus consisted of a funnel (either glass or plastic, depending on the processing method) and a 40-mesh *per* µm brass wire screen positioned approximately 4.00 cm below the top of the funnel. A rubber tube was attached to the bottom of the funnel, and a clamp was secured to the tube approximately 10.00 cm below the funnel. Warm tap water between 23.00 - 28.00 ˚C was poured into the funnel, and a filter containing 3.00 g fecal sample was placed over the wire screen. Morphological identification was used to identify the larvae, and the arithmetic mean of the LPG was determined at each sampling point.

Animals with high or equal infection intensity of 50 larvae *per* g feces were identified[19] and treated orally with albendazole (5.00 mg kg^{-1})[20] or levamisole (7.50 mg kg^{-1}) on the same day. Then, fecal samples were collected from the same group 14 days after the treatment and examined.

The test used in this research to distinguish resistance was fecal egg count reduction test (FECRT) [21] with some modifications for larvae existence or FLCRT. Following the separation of larvae through the Baermann method, a diagnostic key was utilized to distinguish the lung worms' larvae (*D. filaria, P. rufescens*, *M. capillaris* and *C. ocreatus*).[22] The FLCRT was evaluated as follows with some modifications:[21]

FLCRT = 100 (1-14 days post-treatment/pre-treatment)

DNA extraction and polymerase chain reaction (PCR). Lungs infected with adult *D. filaria* were collected from the slaughterhouse. Adult worms genomic DNA was extracted using a commercial DNA extraction Kit (MBST, Tehran, Iran) according to the manufacturer's instructions. The PCR test with four novel primers targeting species-specific regions of the second *internal transcribed spacer* (*ITS2*) and *cytochrome c oxidase subunit 1* (*COX1*) was designed based on data as well as available sequence information in GenBank®.[23-25] In order to amplify the *ITS2* region of the nuclear ribosomal DNA, *NC1* and *BD3R* primers were employed respectively as described formerly.[23,24] The extracted DNA was then stored at – 20.00 ˚C. Furthermore, to strengthen the *COX1* region of mitochondrial DNA, *LCO1490* and *HCO2198* primers were employed.[25] In order to amplify and sequence the *ITS2* region of the nuclear ribosomal DNA and *COX1* region of mitochondrial DNA in *Dictyocaulus* isolates, primers were utilized through PCR as presented in Table 1.

Statistical analyses. The percentage arithmetic mean larvae count reduction surrounded by its 95.00% confidence intervals was calculated with 200 bootstrapping resampling. In addition, we calculated the fecal larvae count reductions in animals with a fecal larvae count of 50 LPG in an Excel (version 15.0; Microsoft Corp., Redmond, USA) spreadsheet according to the formula mentioned above. To evaluate and compare the rate of reduction between the groups treated with albendazole and levamisole for different parasites, the normality of data distribution was first investigated. The curve is normal when all groups are tested together; but, the details in the normality table show that some groups do not have a normal distribution. Data normalization was performed by several transformation methods; but, normal distribution was not achieved. Therefore, comparisons between groups were performed using Mann-Whitney tests. A threshold of $p < 0.05$ was used for these experiments. The existence of resistance to an anthelmintic class can be established when the reduction in fecal egg count after treatment is less than 95.00%, and the lower limit of the 95.00% confidence interval falls below 90.00%. If only one of these criteria is met, resistance is suspected to be present.[11] The analysis was performed with SPSS Software (version 20.0; IBM Corp., Armonk, USA). Comparisons between groups were performed using Mann-Whitney tests. A threshold of $p < 0.05$ was used for these experiments and its level for each row was presented in significance column.

Results

All the samples studied in this research were infected with lung worms. The results indicated that 3.63, 50.86 and 41.90% were infected with *D. filaria*, *M. capillaris* and *P. rufescens*, respectively. The *C. oceratus* was not seen in the studied animals. The results are presented in Tables 2, 3 and 4.

Table 1. Primers used in this study.

Genes	*Primer*	Forward/Reverse	Sequence
ITS2	*NC1*	Forward	*5′-ACGTCTGGTTCAGGGTTGTT-3′*
	BD3R	Reverse	*5′-TATGCTTAAGTTCAGCGGGT-3′*
COX1	*LCO1490*	Forward	*5′-GGTCAACAAATCATAAAGATATTGG-3′*
	HCO2198	Reverse	*5′-TAAACTTCAGGGTGACCAAAAAATCA-3′*

ITS2: *Second internal transcribed spacer*; *COX1*: *Cytochrome c oxidase subunit 1*.

Table 2. Arithmetic means of fecal larvae count of all goats were expressed as larvae *per* g feces *per* treatment being assessed after 0 and 14 days. Data are presented as mean ± standard error of the mean.

Parasite	0 day after treatment		14 days after treatment	
	Albendazole (n = 40)	Levamisole (n = 50)	Albendazole (n = 40)	Levamisole(n = 50)
Dictyocaulus filaria	4.90 ± 2.21	2.36 ± 1.19	2.81 ± 1.40	1.27 ± 0.71
Muellerius capillaris	51.00 ± 13.49	50.72 ± 13.31	32.72 ± 10.01	32.36 ± 9.78
Protostrongylus rufescens	45.27 ±7.86	38.54 ± 8.66	1.09 ± 0.31	0.45 ± 0.20

Table 3. Fecal larvae reductions using the calculation method being determined 14 days after orally treatment with albendazole and levamisole. Data are presented as mean ± standard error of the mean (95.00% confidence interval; lower - upper bounds).

Parasites	Albendazole	Levamisole
Dictyocaulus filaria	16.66 ± 7.42 (3.24 - 34.20)	19.39 ± 8.50 (6.06 - 37.41)
Muellerius capillaris	24.60 ± 7.10 (9.35 - 40.71)	24.63 ± 6.98 (13.90 - 28.47)
Protostrongylus rufescens	88.59 ± 8.88 (70.45 - 98.44)	90.05 ± 9.01 (71.32 - 99.68)

Table 4. Means comparison of fecal larvae reductions using the calculation method being determined 14 days after orally treatment with albendazole and levamisole. Data are presented as mean ± standard error of the mean.

Parasites	Albendazole	Levamisole	Significance
Dictyocaulus filaria	16.66 ± 7.42	19.39 ± 8.50	0.879
Muellerius capillaris	24.60 ± 7.10	24.63 ± 6.98	0.92
Protostrongylus rufescens	88.59 ± 8.88	90.05 ± 9.01	0.15
Cystocaulus ocreatus	0.00 ± 0.00	0.00 ± 0.00	1.00

The results of FLCRT in the examined animals were positive after treatment. The overall efficacy against *Protostrongylus* was 99% and 98% when treated with albendazole and levamisole, respectively. However, low levels of efficacy of these anthelmintics were detected against *Dictyocaulus* and *Muellerius*. Generally, the lung parasites in goats of this region were resistant to albendazole and levamisole.

According the mentioned data in the Table 4, both anthelmintics studied in present study were similarly effective against the lung parasites of goats. Significant differences were detected between the reductions of larvae levels after treatment with these two mostly used anti-parasitic drugs.

Infected lungs were collected from the slaughterhouse and isolated based on PCR method to identify the species and determine the sequence. Sequencing results showed that the identified parasites were 100% similar to the sequences being recorded in the GenBank®. The sequences were registered with the number of SUB12342687 NEWTTB2-BD3R OP935660 for *ITS* and SUB12548224 seq OQ351009 for *COX* in the GenBank®.

In general, considering that the predominant parasitic infections in the respiratory system of goats in this region were *Mullerius* and *Protostrongylus*, and based on the data obtained in this study, resistance to albendazole and levamisole was observed in these parasites. *Dictyocaulus* formed a small number of lung worms in goats, and interestingly, infection with *Cystocaulus* was not observed in the respiratory tract of the sampled animals.

Discussion

It has been about 45 years since the first supply of broad-spectrum anti-nematode drugs, and nowadays there are reports of resistance of nematodes in digestive and respiratory tracts of small ruminants to these drugs, including benzimidazoles, becoming one of the problems of small ruminants breeding in the whole world.[26]

In addition to the economic damage to livestock farmers, this drug resistance has also caused pharmaceutical companies to face major problems because of the costs needed to produce new drugs. The benzimidazole drug family selectively combines with nematode, cestode and trematode beta tubulin and prevents microtubules formation; thus, disrupting cell division and parasite metabolism.[27]

There are different methods to detect the resistance of nematodes against a drug; but, in most parts of the world, resistance is detected based on the reduction of the number of eggs in feces or growth test of larvae before and after treatment.[28]

Parasitic infections are common in small ruminants of the studied area; a study was conducted in Iran in 2000 to determine the seasonal prevalence of pulmonary parasites on 580 goats with random sampling from herds around Urmia, West Azerbaijan province, Iran. The results of this study showed that out of 580 goats, 144 (24.83%) were infected with *D. filaria*, 81 (13.97%) with *M. capillaris,* 243 (41.90%) with *P. rufescens* and 222 (38.28%) with *C. oceratus*.[29] This research is similar to the first part of our research; but, we found a lower respiratory tract infection.

A recent survey was carried out to evaluate the prevalence of anthelmintic resistance of nematodes in communally grazed goats in a semi-arid region of South Africa. The study assessed the efficacies of fenbendazole, levamisole and rafoxanide by conducting FECRT on herds belonging to 10 small-holder goat farmers. The threshold for anthelmintic resistance was set at 80.00% efficacy. Results from the FECRT revealed that all of the tested drugs exhibited more than 80.00% efficacy, indicating that anthelmintic resistance was not observed in the goat population.[30]

Another study was performed to specify the occurrence of anthelmintic resistance on 30 goat farms in Slovakia during the grazing seasons and compare three routine *in vitro* and *in vivo* methods for detecting anthelmintic resistance in field conditions. The FECRT indicated reductions of 69.20 - 86.20% for the single dose and 36.30 - 45.40% for the double dose. The egg hatch test showed that all farms had benzimidazole-resistant nematodes.[31]

The resistance of nematodes to benzimidazole drugs exists everywhere in the world, and it is necessary to think about the way and dosage of these drugs. The resistance of parasitic nematodes of the sheep digestive system to albendazole and fenbendazole was investigated in Saqez, Iran. This study evaluated 90 sheep in three groups and the results indicated that there was a drug resistance in the sheep of that region to albendazole. The results for fenbendazole resistance were however equivocal and they came to conclusion that they were on the threshold of drug resistance.[17]

So far, no reports have been published in Iran about the resistance of goat lung worms to albendazole and levamisole, and this research is the first of its kind. The overall efficacy against *P. rufescens* was 99.00 and 98.00% when treated with albendazole and levamisole, respectively. However, low levels of efficacy of these anthelmintics were detected against *D. filaria* and *M. capillaris*. Generally, the lung parasites in goats of this region were resistant to albendazole and levamisole.

Totally, it can be concluded that the level of drug resistance varies in different parts of the world; but, the frequencies of drug resistance in different parts of the world are not the same, requiring more studies.

The results of this research showed the lack of expected effect of anthelmintic drugs (albendazole and levamisole) in the anthelmintic treatment regimen in the studied goats. The *P. rufescens* showed high resistance to these drugs.

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Conflict of interest

The authors declare no competing interests.

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