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RESEARCH ARTICLE
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Prevalence of drug-resistant gastrointestinal nematodes in an organized sheep farm

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Abstract

Aim: The present study was aimed to determine the resistance against albendazole, fenbendazole, levamisole and closantel in gastrointestinal (GI) nematodes of sheep.

Introduction: Anthelmintics are used traditionally as an integral part of helminthic control strategies for grazing livestock to prevent production losses from parasitic infections. The continuous and indiscriminate use of the same anthelmintics over years together as the sole means of control are now failing due to the emergence of resistance strains of helminths. Resistance to the commonly used anthelmintics in GI nematodes of sheep has become an increasingly widespread problem throughout the world.

Materials and Methods: Fifty-five naturally infected Madras Red lambs of 6-12 months of age were selected and distributed randomly into five treatment groups of 11 animals each. Four groups were treated orally with albendazole (5 mg/kg), fenbendazole (7 mg/kg), levamisole (7.5 mg/kg) and closantel (10 mg/kg) respectively, whereas the fifth group served as untreated control. Fecal samples were collected per rectum of each lamb just prior to treatment (pre-treatment) and on 7, 14, 21 and 28 days post-treatment. The anthelmintic resistance was evaluated by *in vivo* fecal egg count reduction test (FECRT), post-treatment larval culture and *in vitro* egg hatch assay.

Results: In the FECRT, albendazole reduced the faecal egg count by 86.50%, 84.81%, 85.28% and 84.47% respectively for 4 weeks after treatment. Fecal egg count reduction using fenbendazole was 92.64, 93.04, 90.80 and 90.06% respectively for 4 weeks after treatment. The percent efficacy for levamisole and closantel was more than 95%. The post-treatment larval culture contained only *Haemonchus contortus*. In the *in vitro* egg hatch assay, the ED_{50} value for benzimidazole was 0.299 µg albendazole/ml and levamisole showed an ED_{50} value of 0.283 µg/ml.

Conclusion: Our study confirmed the resistance of *H. contortus* to benzimidazole in sheep.

Keywords: benzimidazole resistance, egg hatch assay, fecal egg count reduction test, gastrointestinal nematodes, sheep.

Introduction

The extensive use of anthelmintics for control of gastrointestinal (GI) nematodes has resulted in the development of resistance to one or more of the widely used anthelmintics in many countries [1]. Resistance to anthelmintics by GI nematodes of sheep and goat is a widespread problem.

A recent study performed on anthelmintic worm control practices in Norwegian sheep and goat flocks has indicated the occurrence of under dosing, a lack of anthelmintic class rotation and, in some breeding areas, a high drench frequency, which alone or in combination, are likely to increase the risk for anthelmintic resistance [2]. Further, mixed grazing of sheep and goats has been evoked as a possible risk factor for the spread and emergence of anthelmintic resistance. A number of reports on anthelmintic resistance were documented in many countries [3,4]. In addition, multiple resistances to most of the anthelmintics against GI nematodes have also been detected in many countries [5]. In India, even though many reports have been undertaken to highlight the resistant status among the GI nematodes [6-8], the reports about the same in South India are very limited.

Hence, this study was designed to detect the resistance to the most commonly used anthelmintics viz. albendazole, fenbendazole, levamisole and closantel against GI nematodes of sheep in an organized farm by the widely used *in vivo* fecal egg count reduction test (FECRT) and *in vitro* egg hatch assay.

Materials and Methods

Ethical approval

The study was conducted after the approval of the Institutional Animal Ethics Committee.

In vivo assay - FECRT

The present study was carried out at Livestock Research Station, Kattupakkam, a research unit of Tamil Nadu Veterinary and Animal Sciences University. Madras Red breed of sheep formed the experimental animals for this study, which were managed under semi-intensive system of rearing. Regular deworming was carried out under a scheduled

program viz. once in 3 months. Fifty-five naturally infected Madras Red lambs of 6-12 months of age were randomly distributed into four treatment groups of eleven animals each, and the fifth one was maintained as a control group. Four anthelmintics viz. albendazole (Azole, Vet India, 5.0 mg/kg b.wt), fenbendazole (Fzole Vet India, 7.0 mg/kg b.wt), levamisole hydrochloride (Alved, 7.5 mg/kg b.wt) and Zycloz (Closantel, Zydus Agrovet, 10.0 mg/kg b.wt) were used in the study. All the drugs were administered orally.

A total of 5 g of fecal sample were collected per rectum from each lamb just prior to treatment (Pre-treatment sampling) and then on days 7, 14, 21, 28 after treatment (post-treatment sampling). FECRT was performed for detection of resistance as per the guidelines laid down by WAAVP [9]. Reduction in the egg count of <95% and lower confidence level of <95% compared with the untreated control animals are considered as resistant.

Coproculture and larval identification

After examination of the fecal samples for nematode egg counts, the pooled fecal samples were cultured to determine the species spectrum of mixed GI nematode species. The coproculture was done, and the larvae were identified as described by Ministry of Agriculture, Fisheries and Food [10].

In vitro assay - egg hatch assay

Pooled faecal samples were obtained by mixing several samples collected per rectum from a number of sheep. Eggs were isolated from the feces as per the method described by Coles *et al*. [9] and used in the assay.

Preparation of albendazole stock solution and test protocol

A stock solution of albendazole was prepared by dissolving 50 mg of the pure chemical albendazole (Vet India Pharmaceutical Limited) in 250 ml methanol. Working solutions of the same containing albendazole 200-2 µg/ml were prepared by further dilutions with methanol.

A volume of 10 µl of the working solution of albendazole was pipetted into each well of the tissue culture plate. Two ml distilled water containing a minimum of 200 eggs was added to each well so as to get the final concentration of 1-0.01 µg of albendazole/ml. Assays were conducted in triplicate with a minimum of ten serial concentrations for each. Eggs in distilled water alone and in distilled water containing 10 µg methanol were used as controls. After incubation at 27°C for 48 h, two drops of aqueous iodine were added to each well to prevent further hatching and hatched larvae, and the number of eggs in each well were counted. The percentage of eggs, which had failed to hatch at each drug concentration was calculated. The data were subjected to the arc sin transformation to find out the ED_{50} value as per Rahman [11].

Preparation of levamisole stock solution and test protocol

A stock solution of levamisole was prepared by dissolving 50 mg of the pure chemical levamisole (Vet India Pharmaceutical Ltd.) in 250 ml of demonized distilled water. Testsolutions were then made by standard dilution techniques as per Cawthorne and Whitehead [12].

Suspensions of 0.1 ml containing 70-199 eggs were dispensed into each well of a flat-bottomed microtitration plate. The plate was covered and incubated at 26°C in a saturated atmosphere to prevent evaporation. When the first stage larvae became transparent, 0.1 ml of prepared range of levamisole hydrochloride concentrations (0.05-0.7 µg) was added to each well and incubated for 6 h at 26°C to allow hatching in the control well. The plate was then snap cooled for 5 min and then 0.1 ml of chilled 40% formaldehyde was added to each well. The plate was then held at 4°C for overnight cooling. The percentage of eggs, which had failed at each drug concentration, was calculated as described by Dobson *et al*. [13]. The data were subjected to arcs in transformation to find out the ED_{50} value as per Rahman [11].

Result

The pre-treatment, post-treatment egg count and the per cent reduction in the fecal egg counts are presented in Table-1. The fecal egg count reduction of above 95% was obtained with the drug levamisole and closantel for all the 4 weeks after treatment.

Albendazole had efficacy of 86.50, 84.81, 85.28 and 84.47%, respectively at weekly intervals for 4 weeks after treatment with lower than 95% confidence limits amounting to 82.86, 80.55, 81.14 and 79.57% respectively for 4 weeks. Fenbendazole reduced the egg count by 92.64, 93.04, 90.80 and 90.06% respectively at weekly intervals for 4 weeks after treatment with lower than 95% confidence limits amounting to 89.05, 89.71, 86.01 and 85.22% respectively for 4 weeks. The mean fecal egg count reduction in levamisole treatment group was 98.77, 97.47, 97.55 and 97.52% respectively, whereas in closantel treated group, the fecal egg count reduction was 100% in all the 4 weeks.

The post-treatment (albendazole and fenbendazole) larval culture revealed the presence of *Haemonchus contortus* larvae. However, in the other two groups no larvae were found. The ED_{50} value obtained after arcs in transformation of the data using albendazole as a reference drug in the egg hatch assay was 0.299 µg/ml (Figure-1). The ED_{50} value was 0.283 µg levamisole/ml obtained after arcsin transformation of the data in the study (Figure-2).

Discussion

The results of FECRT in this study confirmed the presence of benzimidazole (albendazole and fenbendazole) resistance confirming the findings of Coles *et al*. [9] who reported that there was resistance, when

Table-1: Mean fecal egg count and per cent fecal egg count reduction

Drugs	Weeks	Arithmetic mean	Variance of counts	Per cent reduction	Variance of reduction (log scale)	Approximate 95% confidence level	
						Upper limit	Lower limit
Albendazole	I	200	6000	86.50	0.0136	89.37	82.36
	II	21.8	7636	84.81	0.0146	88.14	80.55
	III	218	7636	85.28	0.0146	88.50	81.14
	IV	227	10182	84.47	0.0179	88.20	79.57
Fenbendazole	I	109	4909	92.64	0.0375	95.05	89.05
	II	100	4000	93.04	0.0364	95.29	89.71
	III	136	8545	90.80	0.0418	93.95	86.01
	IV	145	8727	90.06	0.0375	93.32	85.22
Levamisole	I	18	1636	98.77	0.4500	99.69	95.15
	II	36	4545	97.47	0.3125	99.19	95.05
	III	36	4545	97.55	0.3125	99.22	95.29
	IV	36	4545	97.52	0.3125	99.21	95.19
Closantel	I	0	0	100.0	0	100.0	100.0
	II	0	0	100.0	0	100.0	100.0
	III	0	0	100.0	0	100.0	100.0
	IV	0	0	100.0	0	100.0	100.0

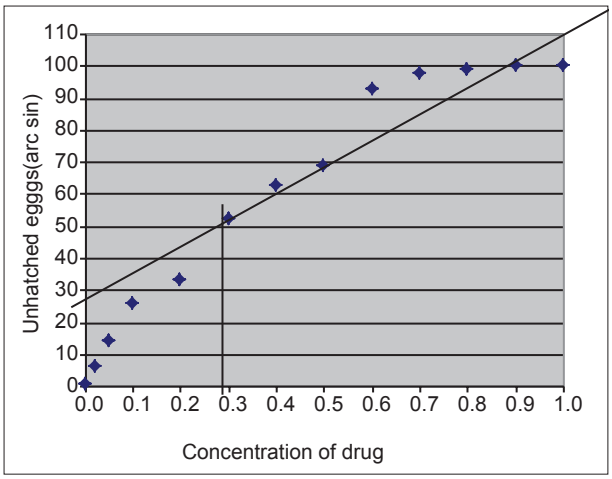

Figure-1: *In vitro* egg hatch assay- albendazole.

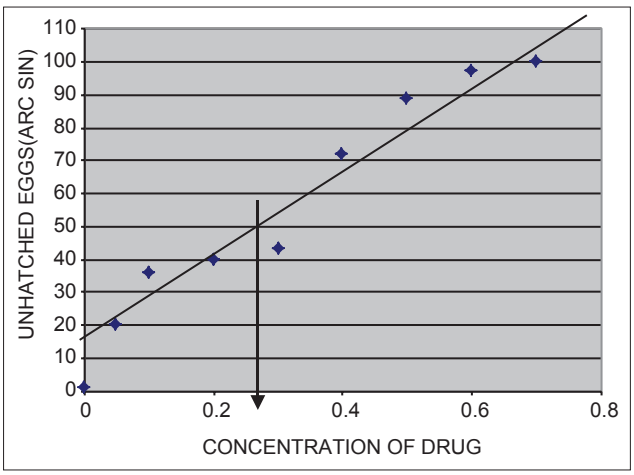

Figure-2: *In vitro* egg hatch assay - levamisole.

an anthelmintic showed efficacy <95% and when the lower confidence limit was <95%. Similar results were also recorded by Yadav *et al*. [14]; Mitchell *et al*. [15] and Jabbar *et al*. [16].

The observation on the fenbendazole resistance in the farm under study was expected since cross-resistance between benzimidazole would equally be reflected in the use of another anthelmintic having similar mode of action [17]. Similar observations were also documented by many workers [18,19].

The survival of *H. contortus* in albendazole and fenbendazole treated groups are in agreement with the findings of Höglund *et al*. [20] and Atle *et al*. [21].

The ED_{50} value of 0.299 µg albendazole/ml obtained in the egg hatch assay for bezimidazole group confirmed resistance as it was well above the limit (0.1 µg/ml) prescribed by Coles *et al*., [9] and the same was higher than the limit of 0.24 µg albendazole/ml as suggested by Smith-Bujis and Borgsteede [22] who also used albendazole as a reference drug in the egg hatch assay. Similar observations on benzimidazole resistance with ED_{50} value >0.10 µg/ml of albendazole were previously recorded by Cawthore and Whitehead [12] in United Kingdom, Easwaran *et al*. [23] in India and Borgsteede *et al*. [24] in The Netherlands.

The ED_{50} value of 0.283 µg levamisole/ml recorded in this study indicated the susceptibility that may be attributed to the fact that levamisole was not used as frequently as that of benzimidazole group of anthelmintics. In earlier studies, Maingi [25] recorded an ED_{50} value of 3.12 µg levamisole/ml indicating resistance.

Since, benzimidazoles are the most widely used anthelmintics, the development of resistance to albendazole was understandable. Frequent using of albendazole in the farm might have contributed to the resistance, since the drug had been used for years together as per the available records which agreed with the findings of Easwaran *et al*. [23], Buttar *et al*. [6] and Acosta *al*. [4].

Conclusion

We conclude that *H. contortus* resistant to benzimidazole was confirmed in this sheep farm. In FECRT, albendazole and fenbendazole reduced the

fecal egg count by <95% for 4 weeks after treatment. This was further confirmed by *in vitro* egg hatch assay with an ED_{50} value of 0.299 µg albendazole/ml. Post-treatment larval culture of albendazole and fenbendazole treated groups of sheep contained only *H. contortus*.

Authors' Contributions

TJH and TA designed the experiment, sample collection and experiment was performed by AMS under supervision of TJH. Manuscript preparation was supervised, reviewed and edited by TJH and TA. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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