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Multiple anthelmintic resistance in gastrointestinal nematodes of sheep in Southern India

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ABSTRACT

The occurrence of anthelmintic resistance on three institutional sheep farms in Tamil Nadu, India was investigated using the Faecal Egg Count Reduction Test (FECRT) for both benzimidazoles and levamisole, the egg hatch assay for benzimidazole (BZD) and Larval Migration Inhibition Assay (LMIA) for levamisole (LEV). FECR value after treatment with albendazole and levamisole ranged from 69-80 and from 70-82 respectively in the three farms examined. The ED_{50} values for TBZ in EHA for isolates from three farms were 0.627, 0.678 and 0.388 µg/mL of TBZ and the LM_{50} values in LMIA for the isolates from three farms were 0.707. 0.437 and 0.377 µg/mL of LEV. The results of the survey indicated multiple resistance in *Haemonchus contortus* and *Teladorsagia* sp. to benzimidazoles and levamisole in farm I, simultaneous resistance in *Teladorsagia sp.* to benzimidazoles and levamisole in farm II and simultaneous resistance in *Haemonchus contortus* to benzimidazoles and levamisole in farm III.

Key words: *Haemonchus contortus, Teladorsagia* sp., benzimidazoles, levamisole, resistance

Introduction

Anthelmintic resistance in gastrointestinal nematodes has become a widespread problem throughout the world in sheep and goats, especially in organized farms (JACKSON, 1993).There has been an increasing number of reports of anthelmintic resistance in nematodes of sheep and goats in India (VARSHNEY and SINGH, 1976; YADAV, 1990; YADAV et al., 1993; SINGH et al., 1995; GILL, 1996; SWARNKAR et al., 1999; DHANALAKSHMI et al., 2003; JEYATHILAKAN et al., 2003). The significance of anthelmintic resistance in gastrointestinal nematodes of sheep and goats in India emphasizes the need for monitoring of resistant worm populations in sheep and goats. Hence, the present study was undertaken

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to monitor the prevalence of resistant nematodes in sheep, which has hitherto not been carried out in this part of India. Effective worm control is threatened by widespread and increasing levels of anthelmintic resistance thereby necessitating alternative control measures. The results of the present survey have indicated the need for strategic drenches in combination with grazing management for slowing down the spread of anthelmintic resistant worm populations in sheep and goats.

Materials and methods

Animals and management. The survey was undertaken in three institutional farms in southern India. In these farms, a semi-intensive type of management was practiced wherein sheep and goats were grazed for at least 6 to 8 hr a day in paddocks of improved grasslands. The flock size in these farms ranged from 150 to 800 animals. Sheep breeds raised included Bharat Merino and Avikalin at CSWRI, Mannavanur (Farm I), Merino, Sandino and Dorset in Sheep Breeding Research Station, Sandynallah (Farm II) and Mecheri at Veterinary College and Research Institute, Namakkal (Farm III). They were housed overnight in raised slatted floor or mud floor pens and they were offered water *ad libitum*, concentrate feed and mineral licks. The routine worm control procedures on all organized farms were to drench 6 times a year, usually with benzimidazoles or imidazothiazole compounds.

Experimental design. In each farm, naturally infected sheep were divided into three groups of 20 animals each. Sheep were not drenched with any anthelmintic for a period of 8 weeks prior to the start of the study. The animals were individually tattooed or ear tagged. Group I animals were treated with albendazole (Valbazen, Pfizer Limited, India) at 10 mg per kg body mass and Group II with levamisole (Helmonil, Alved Pharma and Foods Private Limited, India) at 7.5 mg per kg body mass and Group III animals remained untreated and served as controls. Drenches were administered to all animals with the dosage based on the heaviest animals in each group. Faecal samples were collected from the rectum of each sheep before treatment and after 14 days post treatment with anthelmintics. Faecal cultures before and after treatment were performed on pooled samples from each farm and the infective stage larvae were identified as described (ANONYM., 1986).

Faecal egg count reduction test (FECRT). Faecal egg counts were determined using the sensitive flotation method described by JACKSON (1974). The mean faecal egg count for each treatment group was calculated and compared with that of the control group. The faecal egg count reduction per cent was calculated using the RESO computer programme (MARTIN and WURSTHORN, 1991) and 95 percent confidence intervals were calculated using the arithmetic mean as per the method described by GRIMSHAW et al. (1994).

Egg hatch assay (EHA). EHA was performed using pure thiabendazole (TBZ:Sigma, USA) in dilutions ranging from 0.05 - 0.4 µg/mL as per the method described by JACKSON et al. (2002a). Eggs were recovered from pooled faecal samples after 14 days from treated and control groups. Eggs were extracted by the method described by JACKSON et al. (2002a). Approximately, 100 strongyle eggs were incubated in final concentrations of thiabendazole at 0.05, 0.1, 0.3 and 0.4 µg/mL for 48 hrs at 26 °C in 24 well plates. A control well (2 mL of distilled water) without anthelmintic was included in the test. The incubation was terminated by adding a drop of Lugol's iodine to each well.

Larval migration inhibition assay (LMIA). Larval Migration Inhibition Assay was performed as per the method described by JACKSON et al. (2002b). A stock solution of levamisole (Sigma-Aldrich, USA) was prepared by dissolving levamisole in distilled water followed by dilution in distilled water to give a final stock solution of 1 g in 1000 mL or 1000 ppm. The test was performed in 24 well plates (Nunc). An improved nylon filter apparatus was used.

Data analysis. The percentage reduction in faecal egg count and the 95 per cent confidence intervals were calculated for each treatment group from the arithmetic means of the faecal egg counts. The percentage reduction was calculated as 100 (1-Xt/Xc) where 't' was the mean egg count of the treated group and 'c' was the mean egg count of the untreated group. Resistance to an anthelmintic group was considered to be present if the percentage reduction in egg count was less than 95 per cent and the lower limit of the 95 per cent confidence interval was less than 90%. Data from the EHA and LMIA were analysed to determine the ED_{50} (concentrations of TBZ at which prevent 50% of the eggs fail to hatch in the EHA) and LM_{50} (concentrations of LEV at which 50% of the larvae fail to migrate in LMIA). ED_{50} in excess of 0.1 µg/mL of thiabendazole was considered as resistant. The percentage migration for each concentration was calculated as 100 (1-Nm/ Nm+Nr) where 'Nm" was the number of larvae migrated through mesh and 'Nr' was the number of larvae retained in the mesh. The drug concentration against percentage hatch and migration was plotted and from the graph, ED_{50} and LM_{50} values were arrived.

Results

Faecal egg count reduction test (FECRT). The results of FECRT are presented in Table 1. FECR value after treatment with albendazole and levamisole ranged from 69-80 and from 70-82 respectively in the three farms examined. The most common genus identified in faeces from control animals were *Haemonchus*, *Teladorsagia* and *Cooperia* in Farm I; *Teladorsagia*, *Haemonchus*, *Cooperia* and *Trichostrongylus* in Farm II; *Haemonchus* and *Trichostrongylus* in farm III. In cultures from the treated group, *Haemonchus* and *Teladorsagia* was present in samples in farm I, *Teladorsagia* in farm II and only *Haemonchus* in samples from farm III.

Table 1. Faecal egg count reductions calculated on pre and post anthelmintic treatment egg counts

Farms	Drugs	Arithmetic mean	Percentage of reduction (FECR %)	95% confidence limits	
				Upper limit	Lower limit
I	Albendazole	718	84	90	75
	Levamisole	888	81	88	70
II	Albendazole	915	80	87	69
	Levamisole	775	83	89	74
III	Albendazole	574	88	92	80
	Levamisole	562	88	92	82

Table 2. Gastrointestinal nematode larvae in cultures of faecal samples obtained from sheep before and after treatment

Farms	Before treatment	After treatment
I	*H. contortus*, *Teladorsagia* spp and *Cooperia* spp.	*H. contortus*, *Teladorsagia* spp.
II	*Teladorsagia* spp, *H. contortus* and *Trichostrongylus* spp.	*Teladorsagia* spp.
III	*H. contortus* and *Trichostrongylus* spp.	*H. contortus*

Table 3. ED_{50} values for thiabendazole in the egg hatch assay (EHA) and LM_{50} for levamisole (LEV) in the Larval Migration Inhibition Assay (LMIA) carried out on faecal samples from sheep on 3 institutional farms in Southern India

Farms	ED_{50} for thiabendazole in EHA (µg/mL)	LM_{50} for levamisole in LMIA (µg/mL)
I	0.627	0.707
II	0.678	0.437
III	0.388	0.377

The results indicated that benzimidazole and levamisole resistant isolates of *Haemonchus contortus* and *Teladorsagia* were present in sheep at farm I, benzimidazole and levamisole resistant isolates of *Teladorsagia* sp. in sheep at farm II and benzimidazole and levamisole resistant isolates of *Haemonchus contortus* at farm III.

Egg hatch assay (EHA). In all three institutional farms examined, the ED_{50} values obtained after probit analysis of data in the EHA, ranged from 0.588 to 0.627 µg of thiabendazole/mL demonstrating the presence of benzimidazole resistant nematodes (Table 3 and Fig. 1).

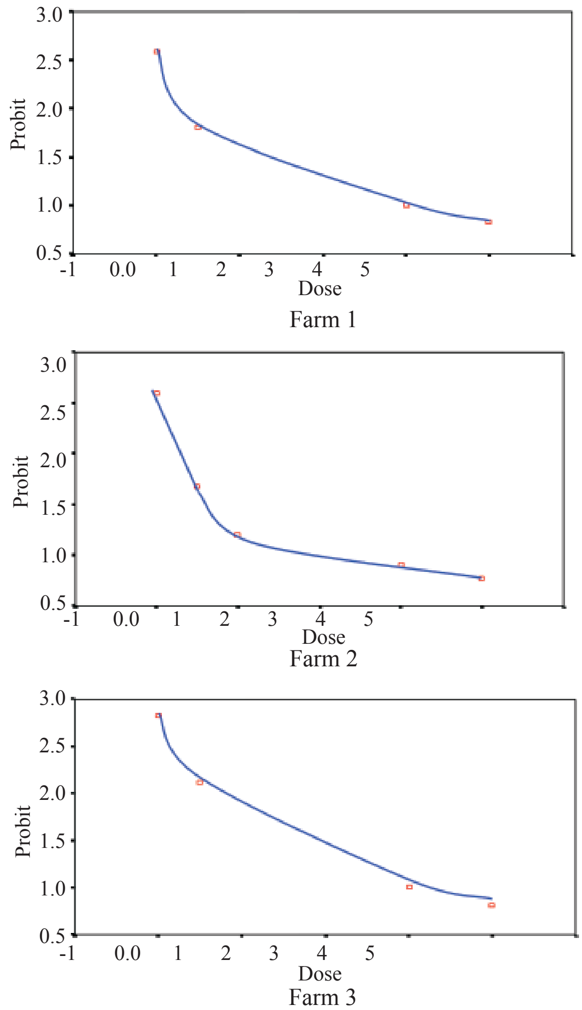

Fig. 1. Probit analysis of egg hatch assay with Thiabendazole in institutional farms

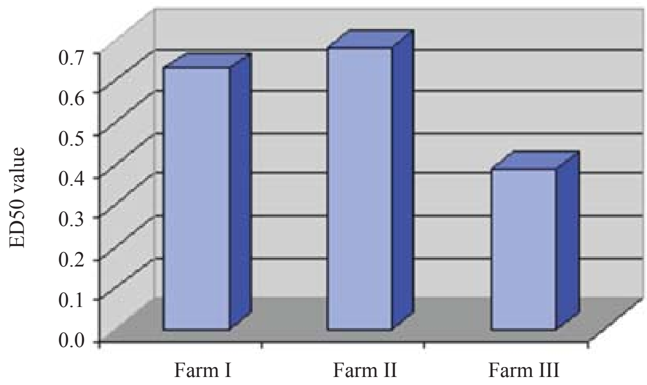

Fig. 2. Comparative anthelmintic resistance by EHA

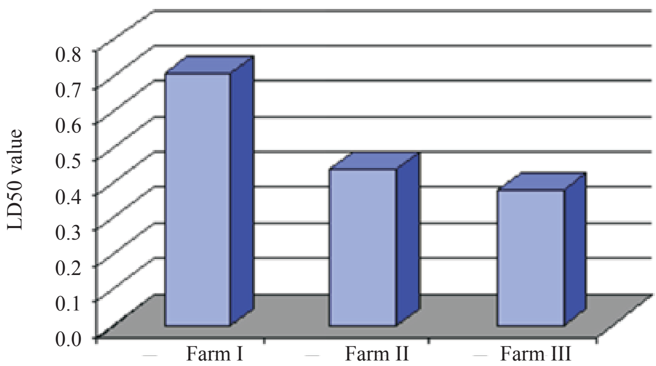

Fig. 3. Comparative anthelmintic resistance by LMIA

Larval migration inhibition assay (LMIA). The concentrations of levamisole required to obtain 50 per cent inhibition of L_3 migration (LM_{50} values) are presented in Table 3. The LM_{50} values for isolates obtained from sheep in the three institutional farms were 0.707, 0.437 and 0.377 µg/mL respectively (Fig. 3) and all these three farms were declared resistant by FECRT. The FECR values and 95% confidence levels were significantly lower for LEV thereby indicating strong resistance to LEV in the gastrointestinal nematodes of sheep in these farms.

Discussion

The results obtained from the FECR test and the *in vitro* assays, EHA and LMIA, indicate the presence of anthelmintic resistance to BZD and LEV in the gastrointestinal nematodes of sheep in three institutional farms in Tamil Nadu, India. This compares well with published reports on anthelmintic resistance in India (VARSHNEY and SINGH, 1976; YADAV et al., 1993; SINGH et al., 1995; GILL, 1996; SWARNKAR et al., 1999; DHANALAKSHMI et al., 2003).

In this study, *Haemonchus contortus* predominated in larval cultures of faeces in two farms (Farm I and III) while *Teladorsagia* sp. was involved in resistance in farm II. In other studies, BZ resistant *H. contortus* (VARSHNEY and SINGH, 1976; YADAV, 1990; DHANALAKSHMI et al., 2003; JEYATHILAKAN et al., 2005) and multiple anthelmintic resistances in *H. contortus* against BZD and tetramisole (SWARNKAR et al., 1999) in sheep were documented. In the present study, multiple anthelmintic resistance in *H. contortus* and *Telodarsagia sp.* against BZD and LEV were recorded. Reports of *H. contortus* (YADAV, 1990; SWARNKAR et al., 1999) resistant to BZD or to LEV and simultaneous resistance to BZD and LEV have been published in India during the past 15 years but to our knowledge, this is the first report of *Telodarsagia* sp showing simultaneous resistance to both BZD and LEV in nematodes of sheep in India.

The egg hatch assay indicated the presence of BZD resistant nematodes in all the three farms examined. In our survey, the ED_{50} ranged from 0.388-0.678 µg/mL and WAAVP recommends that an ED_{50} value in excess of 0.1 µg of TBZ/mL is suggestive of BZD resistance (COLES et al., 1992). In this study, the results from the LMIA were very similar to those from the FECRT and EHA, demonstrating the effectiveness of this alternative in vitro technique for determining anthelmintic efficacy. The LM_{50} values recorded in LMIA ranged from 0.377-0.707 µg/mL.

Resistance to BZD and LEV seems to have developed following prolonged and intensive use of the above anthelmintics in all the three farms. Albendazole, fenbendazole and levamisole are widely used anthelmintics in India and different manufacturers sell them under 8-15 trade names. In all the three institutional farms, albendazole, fenbendazole and levamisole/tetramisole had been widely used over the past 20 years in two farms (I and II) and for 15 years in farm III. BZD and LEV groups of drugs were used until 2005.

The prevalence of nematodes resistant to BZD and LEV in sheep in a farm in Tamil Nadu in Southern India (Farm II) was first reported by GILL (1996) but the author did not mention the species of the resistant nematodes. Later, DHANALAKSHMI et al. (2003) recorded BEZ resistant *H. contortus* in 9 out of 10 farms in Karnataka in Southern India and JEYATHILAKAN et al. (2003) recorded the prevalence of BEZ resistant *H. contortus* in two institutional farms in northern Tamil Nadu in South India. However, this is the first report of simultaneous resistance to both BZD and LEV in two genera of nematodes of sheep in Southern India.

The existence of BEZ and LEV resistant gastrointestinal nematodes in breeding animals from institutional farm increases the risk of dissemination of resistant strains to smallholder farmers' flocks as farm bred sheep and goats are distributed to farmers. Our findings indicate the need for further research into anthelmintic resistance in nematodes of sheep maintained by smallholder farmers and for veterinarians to monitor resistance through periodical estimation of faecal egg counts in sheep.

The results of both FECRT and EHA for BZD in sheep showed that in all the three institutional farms in Southern India, resistant nematodes were present. Similarly, the results of FECRT and LMIA for levamisole in sheep showed a moderate to high degree of resistance to levamisole in the three institutional farms. The degree of resistance to both BZD and LEV was comparatively higher in farms I and II than in farm III and this is presumably due to the fact that in farms I and II, BZD and LEV had been used for more than 25 years whereas farm III is only 15 years old.

In conclusion, the results of the present study, along with the available literature on anthelmintic resistance from other states of India, indicate that anthelmintic resistance in gastrointestinal nematodes of sheep and goats has become a serious problem warranting measures to delay the spread of anthelmintic resistant nematodes in sheep in India.

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SAŽETAK

Pojava otpornosti oblića ovaca na antihelmintike istraživana je na trima farmama u Tamil Nadu u Indiji istražena je testom smanjenja broja jajašaca u izmetu za benzimidazol i levamisol, testom izleženih jajašaca za benzimidazol i testom inhibicije migracije ličinaka za levamisol. Vrijednosti za smanjeni broj jajašaca nakon liječenja albendazolom iznosile su 60 do 80, a nakon liječenja levamisolom 70 do 82. Vrijednosti ED_{50} za tiabendazol u testu izleženih jajašaca za izolate sa svake pojedine farme iznosile su 0,627, 0,678 i 0,388 µg/mL tiabendazola, a vrijednosti LM_{50} u testu inhibicije migracije ličinaka za izolate sa svake od triju farmi iznosile su 0,707, 0,437 i 0,377 µg/mL levamisola. Rezultati pretrage upućuju na višestruku otpornost vrste *Haemonchus contortus* i *Teladorsagia* sp. na benzimidazol i levamisol na farmi I, istodobnu otpornost *Teladorsagia* sp. na benzimidazol i levamisol na farmi II i istodobnu otpornost oblića *Haemonchus contortus* na benzimidazol i levamisol na farmi III.

Ključne riječi: *Haemonchus contortus*, *Teladorsagia* sp., benzimidazol, levamisol, otpornost