RESEARCH ARTICLE

Resistance to Benzimidazoles in *Haemonchus*, *Trichostrongylus* and *Strongyloides* species in Sheep Farms of Three Agro-Climatic Zones of Haryana, India

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Abstract

The regular and frequent use of similar anthelmintics has led to the development and prevalence of anthelmintic resistance which hinders the control of gastrointestinal nematodes (GINs) in sheep. The present research work was planned with the aim of studying the status of benzimidazole group of drug to GINs in sheep farms (one organized and 3 unorganized) of arid (Zone-I, Hisar), semi-arid (Zone-II, Kaithal) and dry sub-humid (Zone-III, Ambala) Zones of Haryana, India using *in vivo* faecal egg count reduction test (FECRT) and *in vitro* egg hatch assay (EHA). The FECRT resulted moderate resistance (60.60-75.26%) in all the selected sheep farms against fenbendazole @ 5 mg/kg b.wt. orally. The faecal culture revealed the predominance of *H. contortus* infective larvae with few *Trichostrongylus* spp. and *Strongyloides* spp. However, EHA revealed resistance in two farms (0.108-0.110 µg TBZ/mL) and susceptibility (0.087-0.092 µg TBZ/mL) of other two farms to thiabendazole, *i.e.,* there ED_{50} values were below 0.1 µg TBZ/mL. This seems to be the first study of anthelmintic resistance against GINs in sheep farms from three agro-climatic zones of Haryana. This basic knowledge on resistance of benzimidazoles group of drug will help in formulating a strategy to control gastrointestinal nematodes in sheep of Haryana.

Key words: Anthelminthic resistance, Benzimidazoles, Egg hatch assay (EHA), Faecal egg count reduction test (FECRT), Haryana, Sheep.

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Introduction

The sheep farming produces wool and meat mainly which in term provides livelihood to poor and landless farmers in Haryana and contribute towards the rural economy. The state of Haryana lies in the northern region (27°39′ to 30°35′ N and 74°28′ to 77°36′ E) of India. Haryana has 0.289 million sheep population as per DAHD, Haryana (2020), *i.e.,* 0.08 % of total livestock. The parasitic infection of gastrointestinal nematodes (GINs) like *Haemonchus contortus*, is a main reason of worry for impaired productivity in the sheep farming system. The control of GINs is mainly by broad-spectrum anthelmintics. There are three broad spectrum anthelmintic classes commonly used for treatment and control of nematodes, *viz.,* benzimidazole, imidothiazole and macrocyclic lactones. However, prolonged and under-overdosing of anthelmintics have led to the development of resistance (Falzon *et al*., 2013). The benzimidazole resistance was first reported in 1964 (Drudge *et al*., 1964). Resistance is generally defined as the ability of an organism to survive doses of drug that would normally kill organisms of the same species and stage (Kotze and Prichard, 2016). There are many reports of anthelmintic resistance from different parts of India (Rialch *et al*., 2013; Kumar and Singh 2016; Dixit *et al*., 2017) as well as from other countries (Santiago-Figueroa *et al*., 2019; Dey *et al*., 2020).

For maintaining the efficacy of the available drugs, regular monitoring of the status of anthelmintic resistance is required, at least once in two years (Rialch *et al*., 2013). The faecal egg count reduction test (FECRT) is *in vivo* test which is recommended by the World Association for the Advancement of the Veterinary Parasitology (WAAVP). The FECRT is easily performed and is applicable in the evaluation of the efficacy of any anthelmintic and gives a direct measure of an anthelmintic efficacy, which is informative to the farmer.

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While WAAVP also recommended *in vitro* tests like egg hatch assay (EHA) which is easy to perform and is recommended for large surveys (Ancheta *et al*., 2004). Therefore, the present study was planned to find out the current status of benzimidazoles group of anthelmintic by FECRT and EHA in sheep farms from three agro-climatic zones of Haryana, India.

Materials and Methods

The study was carried out in Haryana, which is located in northwest India between 27 degree 39′ N to 30 degree 35′ N latitude and between 74 degree 28′ E to 77 degree 36′ E longitude and with an altitude between 700-3600 ft above sea level. At 44,212 sq km, Haryana covers 1.34% of India's geographical area. Haryana is extremely hot in summer at around 45°C (113°F) and mild in winter. The hottest months are May and June and the coldest are December and January.

The samples were collected from three agro-climatic zones of Haryana, *viz.,* arid (Zone-I, Hisar), semi-arid (Zone-II, Kaithal) and dry sub-humid (Zone-III, Ambala). Faecal samples were collected from organized Sheep Breeding Farm, LUVAS, Hisar (SBFL) and unorganized farm of Chirod village, Hisar (CVH) from Zone-I. While in Zone-II and Zone-III samples were collected from Budhakhera village, Kaithal (BVK) and Samlehri village, Ambala (SVA), respectively from unorganized farms.

The status of fenbendazole was studied by *in vivo* FECRT method as described by Coles *et al*. (2006). A total of 30 animals from each sheep flock were selected having egg per gram (EPG) ≥150 and no anthelmintic were administered for last 2-3 months. All the selected animals were divided into two groups, having 15 animals each in treatment and control group. The selected sheep were weighed individually and treated with fenbendazole (Panacur® vet suspension 10% by MSD, Mumbai) @ 5 mg/kg b.wt. orally. While animals in the control group were not administered with any anthelmintic. Faecal egg counts (FECs) of each animal was ascertained on zero and 14th day post-treatment by modified McMaster technique to an accuracy of one egg counted representing 50 EPG. Pooled faecal cultures were kept at 27 ± 2°C for 7 days to recover infective third stage larvae from each group.

The infective larvae were identified as per the criteria of Van Wyk and Mayhew (2013). Faecal egg count reduction percentage and confidence intervals (95%) were determined following the method of the WAAVP using arithmatic mean egg counts. The drug was considered fully effective when it reduced the egg counts by more than 95% and lower confidence limits were higher than 90%. The drug was considered moderately resistant when they reduced the egg counts between 60% to 95%, and considered severely resistant when the reduction in egg counts was below 60% along with lower confidence limits below 90%.

The status of thiabendazole (TBZ) was studied by *in vitro* EHA method as described by Coles *et al*. (2006). The pooled faecal samples were collected from each sheep flock and stored in anaerobic condition (Hunt and Taylor, 1989). Collection of nematode eggs was done with the help of a sieve (0.15 mm aperture, 20 cm diameter) and eggs were finally isolated by using saturated salt solution. Finally, eggs were re-suspended in distilled water with 100-150 eggs/100 µL of water. Dilutions of TBZ (Sigma-Aldrich-T8904) were prepared by dissolving 50 mg of TBZ in 5 mL of dimethylsulphoxide (DMSO) to get stock solution A (10 mg TBZ/mL). From solution A, 1 mL was taken and mixed with 9 mL of DMSO to obtain working solution B (1 mg TBZ/mL). Working concentrations of TBZ (0.01, 0.025, 0.05, 0.1, 0.2, 0.3, and 0.5 µg/mL) were prepared, and 10 µL of each dilution was dispensed into seven separate wells of a microtitre plate, in triplicate. Subsequently, 1890 µL of distilled water and 100 µL of the egg suspension were added to each well. For the control group, 10 µL of DMSO was used in place of TBZ. The plates were incubated at 25 ± 1°C for 48 h in a BOD incubator. After incubation, two drops of 1% iodine solution were added to each well to halt development. Eggs (both embryonated and non-embryonated) and hatched larvae were counted using a binocular microscope with a 10x objective lens. A minimum of 100 eggs and larvae were counted per well.

The data generated were statistically analyzed using IBM SPSS software (Version 27.0). The Chi-square test was applied at 5% or 1% level of significance for FECRT. Logistic regression was worked out for EHA.

Results and Discussion

According to FECRT all the sheep farms in three agro-climatic zones of Haryana, India were moderately resistant to fenbendazole drug. The detailed result of FECRT and larval composition on day zero and 14th of each sheep flock

Table 1: Faecal egg counts pre- and post-anthelmintic (fenbendazole) treatment in sheep farms of three agro-climatic zones of Haryana, India

Zone	Farms	Faecal egg counts on days (Mean ± SE)		Faecal egg counts reduction on 14th day (Post-treatment)		Confidence limits at 95%		Resistance status
		0	14	%	Variance (y^2)	Upper	Lower	
Zone I	SBFL	386.66^{a} ±55.94	173.33^{b}±43.05	60.60	0.08	78.66	27.27	Resistant
	CVH	620^{a}±103.37	160^{b}±44.50	66.66	0.09	82.26	37.35	Resistant
Zone II	BVK	693.33^{a}±80.75	153.33^{b}±60.05	75.26	0.16	89.57	41.31	Resistant
Zone III	SVA	680^{a}±105.19	260^{b}±318.02	62.85	0.11	86.57	11.19	Resistant

Means with the same superscripts are not significantly different (p<0.01).

are summarized in Tables 1 and 2, respectively. The highest and lowest efficacy was observed in BVK unorganized farm (75.26%) and SBFL organized farm (60.60%), respectively. The percentage of larva composition of faecal culture on day zero had predominantly *H. contortus* (73-81%), followed by *Trichostrongylus* spp. (3-11%), *Strongyloides* spp. (5-8%) and *Oesophagostomum* spp. (4-5%). However, the percentage of larva composition on 14th day resulted in predominance of *H. contortus* (91-98%) with few *Trichostrongylus* spp. (1-4%) and *Strongyloides* spp. (1-5%). Our findings coincided with Sharma *et al*. (2015) with 84.46 % efficacy in Hisar of Haryana, Rialch *et al*. (2013) with 69.10% efficacy in sub-Himalyan region, India and Ploegera and Everts (2018) with 73.3% efficacy in the Netherlands. The faecal cultures of each sheep flock showed predominance of *H. contortus* infective larvae in post-treatment samples with few *Trichostrongylus* spp. and *Strongyloides* spp. Similar findings were also reported by Kumar and Singh (2016) in Hisar and Vadlejch *et al*. (2014) in Czech Republic.

The detailed result of EHA using thiabendazole for each sheep flock is summarized in Table 3. Farms that were resistant by EHA were SBFL (zone I) and SVA (zone III) having ED_{50} values 0.108 and 0.110 µg TBZ/mL, respectively. However, gastrointestinal nematodes of CVH (zone I) and BVK (zone II) farms were susceptible to TBZ with ED_{50} values of 0.092 and 0.087 µg TBZ/mL, respectively. Only two farms (SBFL and SVA) were in agreement with FECRT, *i.e.,* showing resistance to TBZ. The present findings are in agreement with Varady *et al*. (2007) in Slovakia (0.108 µg TBZ/mL), Maharshi *et al*. (2011) in Rajasthan (0.103 µg TBZ/mL) and Rialch *et al*. (2013) in sub-Himalayan region of northern India (0.105 µg TBZ/mL). The benzimidazoles like thibendazole and current benzimidazoles, *viz.* albendazole, fenbendazole, oxfendazole, mebendazole were released in the early 1960s and late 1970s, respectively (McKellar and Jackson, 2004). Benzimidazoles act on nematodes at the cellular level, mainly by inhibiting the polymerization of microtubules, eventually causing cell death (Martin, 1997).

The benzimidazole resistance to GINs in organized and unorganized sheep farms has been reported by many workers from India and other countries (Sharma *et al*., 2015; Dixit *et al*., 2017; Arsenopoulos *et al*., 2020). The infective third stage larvae, *i.e. H. contortus* is the main GINs involved in benzimidazole resistance, and development of resistance is faster for *H. contortus* as compared to other nematode genera (Van Wyk, 1990). The basic reason behind the resistance of GINs against benzimidazole in sheep may be due to the frequent and indiscriminate use of benzimidazole with other anthelmintics, easy availability, low cost leading to more government supply in veterinary hospital, *i.e.,* more use of BZs drug, underdosing etc. The prevalence of benzimidazole resistance in all three zones of Haryana and elsewhere in the world tells need for continuous monitoring of anthelmintic status for better control of resistance. The SBFL and SVA sheep farms which were resistant by *in vivo* FECRT were also resistant by *in vitro* EHA. The sheep farms CVH and BVK were susceptible by EHA but resistant by FECRT. The findings were in agreement with Maingi *et al*. (1998). However, our findings differ from Rialch *et al*. (2013) in sub-Himalyan region of northern India, who found higher difference in the result of FECRT when compared to EHA. The difference in results by FECRT and EHA in sheep flocks may be due

Table 2: Anthelmintic (Fenbendazole) effect on different genera of gastro-intestinal nematodes of sheep farms of three agro-climatic zones of Haryana, India

Species	Per cent larval composition on day							
	Zone I				Zone II		Zone III	
	SBFL		CVH		BVK		SVA	
	0	14	0	14	0	14	0	14
Haemonchus spp.	81	93	77	96	73	91	79	98
Trichostrongylus spp.	9	2	3	2	7	4	11	1
Oesophagostomum spp.	5	0	4	0	2	0	4	0
Strongyloides spp.	5	5	6	2	8	5	6	1

Table 3: Egg hatch assay (ED_{50}) values using thiabendazole in sheep farms of three agro-climatic zones of Haryana, India

Zone	Farms	*ED_{50} (µg TBZ/mL)	Confidence limits at 95%	R^2	Thiabendazole / Resistance status
Zone I	SBFL	0.108	0.09-0.35	0.86	Resistant
	CVH	0.092	0.08-0.14	0.84	Susceptible
Zone II	BVK	0.087	0.05-0.09	0.82	Susceptible
Zone III	SVA	0.110	0.15-0.18	0.96	Resistant

*ED_{50} (dose required to prevent 50% of the viable eggs from hatching) value above 0.1 µg TBZ/ml is indicative of resistance.

to the fact that adult worms are examined by FECRT and eggs in EHA. According to von Samson-Himmelstjerna *et al*. (2009), the pharmacokinetics of drug in different hosts and immunological factors also influence the difference in results.

Conclusion

The present findings showed high prevalence of GINs resistance to benzimidazole group of drug with the predominance of *H. contortus* infective larvae with few *Trichostrongylus* spp. and *Strongyloides* spp. by *in vivo* FECRT and *in vitro* EHA anthelmintic resistance methods. It is conclusive that the choice of anthelmintic in farms should be based on history, frequency and status of resistance to a particular drug. This is the first report of moderate anthelmintic resistance against benzimidazole group of drug in sheep farms from three agro-climatic zones of Haryana, India.

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