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Benzimidazole resistance in gastrointestinal nematodes of small ruminants of Uttarakhand

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ABSTRACT

The status of benzimidazole (BZ) resistance in gastrointestinal nematodes (GIN) was studied in a total of four flocks of sheep namely UTs1, UTs2, UTs3 (Uttarkashi district) and Bs1 (Bageshwar district) and four goat flocks namely UTg1, UTg2, (Uttarkashi district) and Bg1 (Bageshwar district) and Ug1 (U.S. Nagar district) of Uttarakhand state using egg hatch assay (EHA) and larval development assay (LDA). EHA detected resistance in two out of eight flocks (UTs3 and Ug1). Arithmetic mean and range of ED_{50} value of susceptible group was found to be 0.043µg/ml and 0.020-0.063µg/ml, respectively, and the same for resistant group were found to be 0.121µg/ml and 0.102-0.141µg/ml, respectively. The arithmetic mean and range of ED_{99} value of susceptible group was recorded as 0.295µg/ml and 0.170-0.500µg/ml, respectively, and that of resistant group was observed to be 2.970µg/ml and 1.794-4.146µg/ml, respectively. The arithmetic mean and range of LC_{50} value of susceptible group was found 0.003µg/ml and 0.002-0.003µg/ml, respectively, and those of resistant group was found 0.0150µg/ml and 0.010-0.011µg/ml, respectively. While the arithmetic mean and range of LC_{99} value of susceptible group was found to be 0.010µg/ml and 0.007-0.011µg/ml, respectively and those of resistant group was observed to be 0.784µg/ml and 0.409-1.159µg/ml, respectively. Pooled coproculture examination revealed the presence of predominantly *Haemonchus contortus,* followed by *Trichostrongylus* spp., *Oesophagostomum* and *Strongyloides,* while infective larvae that survived in wells having higher concentrations of Thiabendazole were all *Haemonchus* with few *Trichostrongylus*. BZ resistance has not been reported earlier against *Trichostrongylus* spp. from Uttarakhand. For effective management of GIN in small ruminants, evaluation of efficacy of anthelmintics is important to regularly monitor anthelmintic resistance. The baseline information thus generated will enable timely management of benzimidazoles resistance in GIN.

Key words: Anthelmintic resistance, Benzimidazole, Gastrointestinal nematodes, Goat, Sheep

In India, small ruminants contribute in providing economic security to small and landless farmers besides serving as a source of animal protein for human consumption. Parasitism is one of the most important causes of production losses in small ruminants around the world. Use of anthelmintics remains the primary control strategy for all important nematodes. Compulsory and often indiscriminate use of these drugs has resulted in emergence of anthelmintic resistance. Efficacy of various anthelmintics must be monitored regularly so that proper selection of anthelmintic can be done otherwise there will be huge economic losses in terms of anthelmintics cost, sustained parasitic load due to ineffective worm control strategies and increased selection of resistant worms. The growing importance of these anthelmintic-resistant nematodes and the need for reliable information on their occurrence and spread has increased the need for standardised tests (Coles *et al*. 1992). Benzimidazole (BZ) resistance has been commonly reported in sheep population of India (Garg and Yadav, 2009) including different northern India (Pandey and Vatsya, 2013, Rialch *et al*. 2014).

To know the status of BZ resistance in different gastrointestinal nematodes (GIN) in sheep and goats, two *in vitro* tests namely egg hatch assay (EHA) and larval development assay (LDA) were employed. *In vitro* tests are cheaper to perform, less time consuming, and therefore more suitable for large surveys. When compared to *in vivo* FECRT, inter-animal variation and the pharmacokinetics of the drug in host does not affect the quality of data. The objective of this study was, therefore, to examine the relationship between the ED_{50} and LC_{50} values obtained using the EHA and the LDA on field isolates of GIN in sheep and goats.

MATERIALS AND METHODS

The status of benzimidazole resistance was studied in a total of four flocks (50-100 animals in each flock) of sheep namely UTs1, UTs2, UTs3 (Uttarkashi district) and Bs1

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(Bageshwar district) and four goat flocks consisting of 30-50 animals in each flock namely UTg1, UTg2, (Uttarkashi district) and Bg1 (Bageshwar district) and Ug1 (U.S. Nagar district) of Uttarakhand state. Uttarkashi and Bageshwar districts are located in hills and U.S. Nagar in Tarai region of the state. Animals selected from each flock were screened for the presence of GIN. Selected animals were not treated with any kind of anthelmintic 8-12 weeks before the commencement of the test and were having epg above 150. For species identification of resistant and susceptible GIN, faecal culture of pooled samples from selected animals of treated and control group was done separately.

Egg hatch assay (EHA): EHA was carried out as described by Le Jambre (1976) with slight modifications (Taylor *et al*. 2002, Coles *et al*. 2006). Faecal samples were collected rectally from animals and pooled. The pooled faecal samples from each flock were either used within 3 h of collection or were stored anaerobically as described by Hunt and Taylor (1989). The samples were used within 7 days of collection. Nematode eggs were collected by sieving through a 0.15mm aperture, 20 cm diameter sieve and then finally recovered by using saturated salt solution (MAFF 1986). Eggs were re-suspended in deionised water with 100-150 eggs/100µl of water. 10 µl of prepared dilutions of thiabendazole (TBZ): 0.01, 0.025, 0.05, 0.1, 0.2, 0.3, 0.5 µg/ml (von Samson- Himmelstjerna *et al.* 2009) were added to seven different wells. DMSO was added to one well and kept as control. Each concentration of TBZ was tested in triplicate. 100 µl of egg suspension containing about 100-150 fresh eggs were placed in each well containing TBZ or DMSO in the 24 multiwell plate. 1.890 ml of deionised water was added in each well to make the total volume as 2ml. Outer rows of wells were filled with deionised water. Plates were incubated at 27ºC for 48 hours. Two drops of lugol's iodine were added to each well. Hundreds of the remaining eggs and hatched larvae were counted under compound microscope one by one for each well by carefully washing the contents of the wells onto microscopic slide.

Larval development assay (LDA): Larval development assay was performed as per Hubert and Kerboeuf (1992) with slight modifications. Serial dilutions of TBZ concentrations were made as for EHA ranging from 0.001 to 0.05µg/ml. 10µl of DMSO (in control well) or 10µl of serial dilution of TBZ were added to the bottom of the well. Each concentration of TBZ was tested in triplicate. 100µl of eggs suspension (as prepared for EHA) was added. 10 µl of amphotericin B (0.3mg/ml) and 10µl of yeast extract were added as described by Hubert and Kerboeuf (1984). Final volume of each well was made 2ml by adding deionised water. Outer rows of wells were filled with deionised water. Plates were placed in a small container with open water source for stable humidity and were incubated at 27ºC for 7 days. Number of live third stage larvae were counted in each well.

Statistical analysis: Log probit analysis using SPSS version 17.0 was employed to determine ED_{50} and ED_{99} values (dose required to prevent 50% and 99% of the viable eggs from hatching, respectively) for eggs in EHA and for

Table 1. ED_{50} and ED_{99} (µg/ml) of thiabendazole in EHA and resistance status

Flock no.	ED_{50}(µg/ml)			ED_{99}(µg/ml)			Resistance status*
	ED_{50}	Lower limit	Upper limit	ED_{99}	Lower limit	Upper limit	
UTs1	0.042	0.03	0.56	0.170	0.11	0.46	S
UTs2	0.039	0.03	0.56	0.189	0.11	0.65	S
UTs3	0.102	0.08	0.14	1.794	0.96	4.97	R
Bs1	0.049	0.03	0.08	0.221	0.12	1.53	S
Ug1	0.141	0.10	1.99	4.146	1.79	18.04	R
UTg1	0.048	0.04	0.06	0.247	0.16	0.57	S
UTg2	0.020	0.01	0.03	0.500	0.30	1.26	S
Bg1	0.063	0.04	0.09	0.441	0.25	1.27	S

*R, Resistant; S, susceptible.

Table 2. Arithmetic means and range of ED_{50} and ED_{99} (µg/ml) of thiabendazole in EHA for susceptible and resistant populations of gastrointestinal nematodes

Species	Resistance status	ED_{50} (µg/ml)		ED_{99} (µg/ml)	
		Mean	Range	Mean	Range
Sheep	Susceptible	0.043	0.039-0.042	0.718	0.170-0.221
	Resistant	0.102	0.08-0.14	1.794	0.96-4.97
Goat	Susceptible	0.044	0.020-0.063	0.396	0.247-0.500
	Resistant	0.141	0.10-1.99	1.146	1.79-18.04
Both	Susceptible	0.043	0.020-0.063	0.295	0.170-0.500
	Resistant	0.121	0.102-0.141	2.970	1.794-4.146

LDA, LD_{50} and LD_{99} values (the concentration of TBZ that inhibits development to L_3 stage by 50% and 99%, respectively). Benzimidazole resistance was confirmed by EHA if eggs had ED_{50} value in excess of 0.1µg of thiabendazole per ml (Coles *et al*. 1992). Range and arithmetic mean for LD_{50} and LD_{99} in LDA were described for susceptible and resistant flocks.

RESULTS AND DISCUSSION

Egg hatch assay (EHA): EHA detected resistance in two out of eight flocks screened with ED_{50} value greater than 0.1 µg/ml while six flocks were detected susceptible for benzimidazole (Table 1). All the flocks screened were divided into 2 categories based on their resistance and susceptibility status as determined by EHA and average ED_{50} and ED_{99} values were calculated. Arithmetic mean and range of ED_{50} value of susceptible group was found to be 0.043µg/ml and 0.020-0.063µg/ml, respectively, and the same for resistant group were found to be 0.121µg/ml and 0.102-0.141µg/ml, respectively. The arithmetic mean and range of ED_{99} value of susceptible group was recorded as 0.295µg/ml and 0.170-0.500µg/ml, respectively, and that of resistant group was observed to be 2.970µg/ml and 1.794-4.146µg/ml, respectively (Table 2).

Larval development assay (LDA): All the flocks screened were divided into two categories based on their resistance and susceptibility status as determined by EHA and average LC_{50} and LC_{99} values by LDA were established (Table 3). The arithmetic mean and range of LC_{50} value of susceptible group was found 0.003µg/ml and 0.002-0.003µg/ml, respectively, and those of resistant group was found 0.0150µg/ml and 0.010-0.011µg/ml, respectively. While the arithmetic mean and range of LC_{99} value of susceptible group was found to be 0.010µg/ml and 0.007-0.011µg/ml, respectively and those of resistant group was observed to be 0.784µg/ml and 0.409-1.159µg/ml, respectively (Table 4).

Pooled coproculture examination revealed the presence of predominantly *Haemonchus contortus,* followed by *Trichostrongylus* spp., *Oesophagostomum* and *Strongyloides,* while infective larvae that survived in wells having higher concentrations of TBZ were all *Haemonchus* with few *Trichostrongylus*. BZ resistance has not been reported earlier against *Trichostrongylus* spp. from Uttarakhand.

The EHA is recommended by WAAVP (Coles *et al*. 1992) and is the most widely used *in vitro* method for the detection of BZ resistance in field flocks of sheep and goats (Varady *et al.* 2006). The EHA is based on the ability of eggs of resistant populations of GIN to embryonate and hatch in higher concentrations of BZ than eggs from susceptible populations of GIN (Coles *et al*. 1992, 2006). The results are interpreted using ED_{50} or ED_{99} values (drug concentrations producing 50% and 99% inhibition of hatching in the test, respectively). However, this test detects BZ resistance only if $\geq$25% resistant worms are present in a population (Martin *et al.* 1989). The delineation dose of

Table 3. LC_{50} and LC_{99} (µg/ml) of thiabendazole in LDA and resistance status of gastrointestinal nematodes

Flock no.	LC_{50}(µg/ml)			LC_{99}(µg/ml)			Resistance status*
	ED_{50}	Lower limit	Upper limit	ED_{99}	Lower limit	Upper limit	
UTs1	0.003	0.002	0.003	0.019	0.011	0.051	S
UTs2	0.002	0.001	0.003	0.012	0.007	0.029	S
UTs3	0.011	0.005	0.023	0.409	0.102	31.374	R
Bs1	0.003	0.002	0.003	0.016	0.010	0.039	S
Ug1	0.010	0.005	0.021	1.159	0.213	164.640	R
UTg1	0.003	0.002	0.004	0.016	0.009	0.044	S
UTg2	0.003	0.003	0.004	0.020	0.013	0.044	S
Bg1	0.003	0.002	0.004	0.018	0.011	0.052	S

*R, Resistant; S, susceptible; *, resistance status as per EHA.

Table 4. Arithmetic means and range of LC_{50} and LC_{99} (µg/ml) of thiabendazole in LDA for susceptible and resistant populations of gastrointestinal nematodes

Species	Resistance status	LC_{50} (µg/ml)		LC_{99} (µg/ml)	
		Mean	Range	Mean	Range
Sheep	Susceptible	0.0027	0.002-0.003	0.016	0.012-0.019
	Resistant	0.011	0.005-0.023	0.409	0.102-31.374
Goat	Susceptible	0.003	0.002-0.004	0.018	0.016-0.020
	Resistant	0.010	0.005-0.021	1.159	0.213-164.640
Both	Susceptible	0.003	0.002-0.003	0.010	0.007-0.011
	Resistant	0.015	0.010-0.011	0.784	0.409-1.159

0.1µg/ml of TBZ in EHA provides a good estimate of genotypic resistance (Cudekova *et al.* 2010).

LDA is based on ability of GIN larvae to survive and develop in different concentrations of BZ. In this assay, eggs isolated from faeces are incubated for 7 days in medium after which the percentage of infective and non infective larvae along with unembryonated eggs is calculated for each drug concentration and drug less controls. LDA can detect BZ resistance in GIN of sheep and goats even when 4% resistant individuals are present in a population (Varady *et al.* 2007).

Higher ED_{50} values of BZ were observed in goat flocks as compared to sheep flocks. The same have also been reported by Gallidis *et al.* (2009). This can be attributed to rapid hepatic metabolism of BZ in goats or short lived plasma levels of the active component of drug i.e. lower bioavailability (Swan and Gross 1985, McKenna and Watson 1987). This in turn is compensated for by administrating higher doses of BZ almost twice in goats than sheep. This resulted in development of BZ resistance in goats at a faster rate than in sheep (Charles *et al.* 1989, Varady *et al.* 2011). Twice the recommended dosage of BZ for sheep had been used in goats by several workers (Maingi *et al.* 1998).

BZ resistance was observed in semi-intensively managed sheep (UTs3) and goat (Ug1) flocks. These flocks had the history of fenbendazole being used for the last 6-8 years. Besides, the animals were drenched 4-5 times a year. Laha *et al.* (1999) also recorded fenbendazole resistance in goats at Mukteshwar, a place located 2,350 m above mean sea level in Uttarakhand state. Lack of awareness of usage of fenbendazole at twice the recommended dose in goats and practice of treating the animals without knowing the appropriate body weight of animals might have contributed to development of fenbendazole resistance.

The LC_{50} values for BZ resistant GIN populations in LDA could not be established either in sheep or goat flocks. However, the LC_{50} values obtained in LDA were lower than EHA, suggesting that probably GIN larvae are more sensitive to the action of BZs as compared to eggs. Similar observations have been made by Hubert and Kerboeuf (1992) and Craven *et al.* (1999). The pharmacokinetics of drug in different hosts and immunological factors influenced the difference in results (von Samson – Himmelstjerna *et al.* 2009). The results of the study indicated that EHA and LDA could be used to detect BZ in GIN of sheep and goats.

For effective management of GIN in small ruminants, evaluation of efficacy of anthelmintics is important to regularly monitor anthelmintic resistance. In FECRT assay, the faecal samples are to be collected 10 days post treatment. However, this becomes difficult in the event of heavy rainfall/snowfall and landslides in hilly areas of Uttarakhand. In such situations, *in vitro* assays namely EHA and LDA could be used to detect BZ resistance in GIN of sheep and goats in these regions. The baseline information thus generated will enable timely management of benzimidazoles resistance in GIN.

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REFERENCES

Charles T P, Pompeu J and Miranda D B. 1989. Efficacy of three broad-spectrum anthelmintics against gastro-intestinal nematode infections of goats. *Veterinary Parasitology* **34**: 71–75.

Coles G C, Bauer C, Borgsteede F H, Geerts S, Klei T R, Taylor M A and Waller P J. 1992. World Association for the Advancement of Veterinary Parasitology (WAAVP) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology* **44:** 35–44.

Coles G C, Jackson F, Pomroy W E, Prichard R K, von Samson-Himmelstjerna G, Silvestre A, Taylor M A and Vercruysse J. 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology* **136**: 167–85.

Craven J, Bjorn H, Barnes E H, Henriksen S A and Nansen P A. 1999. A comparison of *in vitro* tests and a faecal egg count reduction test in detecting anthelmintic resistance in horse strongyles. *Veterinary Parasitology* **85**: 49–59.

Cudekova P, Varady M, Dolinska M and Konigova A. 2010. Phenotypic and genotypic characterisation of benzimidazole susceptible and resistant isolates of *Haemonchus contortus. Veterinary Parasitology* **172**: 155–59.

Gallidis E, Papadopoulos E, Ptochos S and Arsenos G. 2009. The use of targeted selective treatments against gastrointestinal nematodes in milking sheep and goats in Greece based on parasitological and performance criteria. *Veterinary Parasitology* **164**: 53–58.

Garg R and Yadav C L. 2009. Genotyping of benzimidazole susceptible and resistant alleles in different populations of *Haemonchus contortus* from Himalayan and sub-Himalayan regions of North-West India. *Tropical Animal Health Production* **41**:1127–31.

Hubert J F and Kerboeuf D. 1984. A new method for culture of larvae used in diagnosis of ruminant gastrointestinal strongylosis: comparison with faecal cultures. *Canadian Journal of Comparative Medicine* **48**: 63–71.

Hubert J F and Kerboeuf D. 1992. A microlarval development assay for the detection of anthelmintic resistance in sheep nematodes. *Veterinary Record* **130**: 442–46.

Hunt K R and Taylor M A. 1989. Use of the egg hatch assay on sheep faecal samples for the detection of benzimidazole resistant worms. *Veterinary Record* **125**: 153–54.

Laha R, Haemaprasanth, Harbola P C. 1999. Anthelmintic resistance in Pashmina (cashmere) producing goats in India. *Veterinary Research Communication* **23:** 187–89.

Le Jambre L F. 1976. Egg hatch as an *in vitro* assay of thiabendazole resistance in nematodes. *Veterinary Parasitology* **2**: 385–91.

MAFF. 1986. *Manual of Veterinary Parasitology Laboratory Techniques*. 3rd edn, 160 pp. Ministry of Agriculture, Fisheries and Food. ADAS, HMSO, UK.

Maingi N, Bjorn H and Dangolla A. 1998. The relationship between faecal egg count reduction test and the lethal dose

50% in the egg hatch assay and larval development assay. *Veterinary Parasitology* **77**: 133–45.

Martin P J, Anderson N and Jarrett R G. 1989. Detecting benzimidazole resistance with faecal egg count reduction tests and *in vitro* assays. *Australian Veterinary Journal* **66**: 236–40.

McKenna P B and Watson T G. 1987. The comparative efficacy of four broad spectrum anthelmintics against some experimentally induced trichostrongylid infections in sheep and goats. *Newzealand Veterinary Journal* **35**: 192–95.

Pandey N and Vatsya Stuti. 2013. Detection of Benzimidazole resistance in *Haemonchus contortus* populations collected from Uttarakhand using allele specific PCR assay. *Indian Journal of Animal Sciences* **83** (1): 24–26.

Rialch A, Vatsya Stuti and Kumar R R. 2013. Detection of benzimidazole resistance in gastrointestinal nematodes of sheep and goats of sub-Himalyan region of northern India using different tests. *Veterinary Parasitology* **139**: 312–18.

Swan G E and Gross S J. 1985. Efficacy of ivermectin against induced gastrointestinal nematode infections in goats. *Veterinary Record* **117**: 147–49.

Taylor M A, Hunt K R and Goodyear K L. 2002. Anthelmintic resistance detection methods: a review. *Veterinary Parasitology* **103**: 183–94.

Varady M, Cernanska D and Corba J. 2006. Use of two *in vitro* methods for the detection of anthelmintic resistant nematode parasites on Slovak sheep farms. *Veterinary Parasitology* **135**: 325–31.

Varady M, Cudekova P and Corba J. 2007. *In vitro* detection of benzimidazole resistance in *Haemonchus contortus*: Egg hatch test versus larval development test. *Veterinary Parasitology* **149:** 104–10.

Varady M, Papadopoulos E, Dolinska M and Konigova A. 2011. Anthelmintic resistance in parasites of small ruminants: sheep versus goat. *Helminthologia* **48**: 137–44.

von Samson-Himmelstjerna G, Coles G C, Jackson F, Bauer C, Borgsteede F, Cirak V Y, Demeler J, Donnan A, Dorny P, Epe C, Harder A, Hoglund J, Kaminsky R, Kerboeuf D, Kuttler U, Papadopoulos E, Posedi J, Small J, Varady M, Vercruysse J and Wirtherle N. 2009. Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes. *Parasitology Research* **105**: 825–34.