



Reversion towards benzimidazole susceptibility in *Haemonchus contortus* by resistance management strategies

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Sheep are usually infected with a variety of gastrointestinal nematodes (GINs) through ingestion of the infective larvae of the parasites on the contaminated pasture. The total annual cost of parasitism in sheep in India was estimated to \$ 103 million (McLeod 2004). Singh *et al.* (2011) estimated that under natural challenge of infection in sheep, the components of losses were reduced mutton production (59.56%), increased susceptibility for mortality (16.57%), premature culling (11.25%), reduced fertility (7.97%) and decreased wool yield (4.65%). Since long, anthelmintics are the cornerstone of GIN management programmes. However, worldwide emergence of anthelmintic resistance in nematode parasites had a considerable impact on chemotherapeutic based worm management programmes (Miller *et al.* 2012). The frequency of resistance genes increases as the population is repeatedly exposed to drugs, but whether the converse is true (i.e. a reversion to susceptibility, over time, once selection pressure is removed) remains unclear. It was demonstrated that resistance to the benzimidazole (BZ) remained high for many years even after cessation of BZ use (Borgsteede and Duyn 1989) due to genetic fixation in the worm population (Leathwick *et al.* 2015). However, importance of time length in reversion to susceptibility of a drug resistant population had been documented and partial reversion to susceptibility to fenbendazole (FBZ) was observed in FBZ resistant *Haemonchus contortus*, after 10–12 years of its discontinuation (Das and Singh 2010). Further, level of refugia has been considered as the single most important factor contributing to selection for anthelmintic resistance parasites (van Wyk 2001). The refugia based strategies like targeted selective treatment (Singh *et al.* 2015), community dilution of resistant population (Singh and Swarnkar 2017) and administration of anthelmintics as per climatic conditions (Hodgkinson *et al.* 2019) could conserve susceptible alleles within the parasite population. The resulting mixtures of resistant and susceptible genotypes on pasture have potential for cross-breeding and/or diluting the

frequency of resistant genotypes within a worm population. The present study was aimed to observe the impact of withdrawal of anthelmintic type and increase in refugia through community dilution on reversion towards BZ susceptibility in *H. contortus* at an organized sheep farm in semi-arid Rajasthan.

The study was carried out at ICAR-Central Sheep and Wool Research Institute, Avikanagar located in semi-arid region of Rajasthan (India). The climatic conditions suitable for translation of exogenous stages *H. contortus* prevails from June to mid-September in the study area (Swarnkar and Singh 2011). At the farm, emergence of BZ resistance in *H. contortus* was first noticed in the year 1995 with 80% faecal egg count reduction on faecal egg count reduction test (FECRT) and ED₅₀ value of 0.239 µg TBZ/ml on egg hatch assay (EHA) (Singh *et al.* 1995) and use of BZ anthelmintics was discontinued from the year 1996. Thereafter, the status of BZ resistance was assessed by FECRT, EHA and polymerase chain reaction (PCR) assay. The efficacy of BZ anthelmintics varied between 0–44% in the year 2000 (Swarnkar *et al.* 2001) with predominance of BZ-resistant alleles (>90%) between the year 2005 to 2010 (Tiwari *et al.* 2006, Maharshi *et al.* 2011). The efficacy was still observed low (26% on FECRT, ED₅₀ of 0.196 µg TBZ/ml on EHA) even after 15 year post BZ withdrawal. From the year 2010 onward, the concept of community dilution was implemented by allowing contamination of farm pasture with animals (purchased and tested for anthelmintic resistance in quarantine) harbouring BZ susceptible *H. contortus*. In addition, concept of refugia was also taken in consideration by shifting strategic drench during early monsoon to mid-late monsoon (Singh and Swarnkar 2017).

In the month of July–August, 2018, the status of anthelmintic resistance in *H. contortus* was evaluated by employing *in vivo* FECRT in four flocks of Malpura, Patanwadi and Avishaan breeds as per Coles *et al.* (1992) using fenbendazole (@ 5.0 mg/kg body weight), levamisole (@ 15 mg/kg body weight) and closantel (@ 10 mg/kg body weight). The faecal egg counts for each animal were estimated by modified McMaster technique (MAFF 1986)

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on day 0 (pre-treatment) and 12th (post-treatment). Per cent faecal egg count reduction (% FECR) was calculated by RESO calculator (Martin and Wurstthorn 1991). Resistance to anthelmintic was considered present if (i) the % FECR was less than 95 and (ii) the lower 95% confidence limit was < 90. If only one of these two criteria was met, the resistance was suspected.

In the month of August 2018 and July 2019, EHA was performed on eggs separated from faeces collected from five flocks of Malpura, Patanwadi, Avikalin and Avishaan breeds using pure thiabendazole (TBZ) in a dilution series of 0.0625 to 1 µg TBZ/ml (Coles *et al.* 1992). The data were analysed by probit analysis to obtain ED₅₀ value for egg hatch. ED₅₀ value in excess of 0.1 µg TBZ/ml was suggestive of BZ resistance. Simultaneously, individual 3rd stage larvae of *H. contortus* isolated from coproculture were genotyped to ascertain the proportion of different genotypes in supra-population with respect to BZ resistance through allele specific-polymerase chain reaction (AS-PCR) technique. The genomic DNA was extracted as per Silvestre and Humbert (2000). The primers used for amplification were common reverse primer (TGG 312): 5'-GGAACCA-TGTTACCGGCTAAC-3', susceptible primer (CAW 106): 5'-TAGAGAA CACCGATGAAACATT-3' and resistant primer (TGG 331): 5'-GTAGAGAACACCGATGAAA-CATA-3' (Winterrowd *et al.* 2003). The PCR reaction mixture comprised 20 µl volume as 1X *Taq* polymerase buffer (2 µl), 0.5 U *Taq* DNA polymerase (0.5 µl), 200 µM dNTP's mixture (1.6 µl), 1.5 mM MgCl₂ (0.6 µl), 200 nM of each primer (0.4 µl each), template DNA (2.0 µl) and mili Q water (12.5 µl). PCR reaction conditions were optimized using initial denaturation at 95°C for 10 min followed by 50 cycles of denaturation at 95°C for 30 sec, annealing at 61°C for 30 sec, extension at 72°C for 60 sec and final extension at 72°C for 5 min. Analysis of amplified PCR products was done on 1.5% agarose gel stained with ethidium bromide. Frequencies of different genotype and alleles were calculated as per Pierce (2003).

Following the implementation of the community dilution strategy and manipulating the refugia from the year 2010

onward, all the flocks showed <95% efficacy (with lower 95% confidence limit of <90) for fenbendazole against *H. contortus* in the year 2018 (22 year post BZ withdrawal) suggesting persistence of BZ resistance in *H. contortus*. However, a significant improvement (86–93%) in efficacy of BZ was noticed against *H. contortus* (Table 1) as compared to earlier results with discontinued use of BZ only (80% in 1995, 0–44% in 2000, 26% in 2010). Other anthelmintics, viz. levamisole and closantel showed 96 to 100% efficacy suggesting absence of resistance to these classes of anthelmintics.

The results of *in vitro* EHA in a total of 14 samples from five flocks during 2018–20 showed mean ED₅₀ value of 0.075±0.008 µg TBZ/ml (ranging from 0.011 to 0.119 µg TBZ/ml) suggesting BZ susceptibility in *H. contortus*. A significant reduction in ED₅₀ value was observed following implementation of community dilution and refugia manipulation strategies as compared to only strategy of discontinued use of BZ (0.239±0.013 µg TBZ/ml in 1995, 0.196±0.011 µg TBZ/ml in 2010). In similar line, on AS-PCR assay, an increase in proportion of susceptible allele (48%) was observed in infective larvae (L₃) of *H. contortus* during the period 2018–2020 as compared to <10% (7.0% in 2005–06, 5% in 2006–07, 1.6% in 2010–11) prior to implementation of community dilution and refugia manipulation strategies.

As anthelmintics are an integral component of most worm control programmes, much attention has been given to optimizing their use, both in terms of parasite control (Barger 1999) and management of resistance (Dobson *et al.* 2011). Similar to present observations, ineffectiveness of the strategy involving only long-term discontinued use of anthelmintics in reversion to susceptibility has been also reported by Borgsteede and Duyn (1989). However, implementation of different resistance management strategies resulted in positive trend in efficacy of BZ anthelmintics as reflected by results of all the three assays in present study. Like-wise, Leathwick *et al.* (2015) observed that almost exclusive use of combination anthelmintics in conjunction with other resistance management strategies

Table 1. Mean efficacy (%) of anthelmintics against *Haemonchus contortus* in naturally infected sheep

Anthelmintic	Breed (Flock)	FECR (%)	95% Confidence limit		Result
			Lower	Upper	
Fenbendazole	Malpura (1)	90	72	97	Resistant
	Malpura (2)	86	55	95	Resistant
	Patanwadi (3)	93	85	97	Resistant
	Avishaan (4)	92	73	97	Resistant
Levamisole	Malpura (1)	96	91	98	Susceptible
	Malpura (2)	99	90	98	Susceptible
	Patanwadi (3)	99	93	98	Susceptible
	Avishaan (4)	99	95	98	Susceptible
Closantel	Malpura (1)	100	99	100	Susceptible
	Malpura (2)	100	97	100	Susceptible
	Patanwadi (3)	100	99	100	Susceptible
	Avishaan (4)	100	99	100	Susceptible

had prevented the further escalation in anthelmintic resistance. They observed that across the 7 farms, the efficacy of albendazole against *Teladorsagia circumcincta* averaged 66% in year one but at the end of 5 years, it increased to average of 90% and ranged from 71 to 100%. Thus, there was evidence for reversion towards susceptibility in *T. circumcincta*. Similarly, reversion towards susceptibility of fenbendazole and morantel resistant *H. contortus* after withdrawal of these drugs for 10 years was reported in farm flocks at Haryana (Das and Singh 2010). Singh and Gupta (2009) reported partial reversion to susceptibility of fenbendazole and levamisole resistant strain of *H. contortus* on an organized sheep farm after switching over to ivermectin and closantel for 12 years. Recently, Muchiut *et al.* (2019) reported recovery of FBZ efficacy against a resistant population of *H. contortus* by means of refugia management and subsequent introduction of susceptible population. The possible reason for enhanced efficacy could be that any initial fitness cost associated with resistant genotypes would have been eroded by re-association of resistance and fitness traits during the selection process (Prichard 1990). Further, the size of the worm population in refugia was also important, with resistance being delayed more and reversion occurring at lower fitness costs when a larger proportion of the worm population was not exposed to treatment (Maingi *et al.* 1990).

The timing of anthelmintic intervention in flocks should also be considered as an important factor in enhancing the quantum of refugia. Anthelmintic administration should be coordinated with the weather (Barger 1999), as there will be little or no exposure to infective larvae during hot, dry weather. With the occurrence of monsoon, inactive larvae become active and leading to exponential rise in larvae exposure. In semi-arid agroclimatic conditions, period from September to November was appropriate for increasing the frequency of BZ-susceptible alleles in the refugia (Swarnkar *et al.* 2012). Thus, the study indicates the possibility of reversion to BZ susceptibility in *H. contortus* population in farm area with implementation of community dilution and refugia based worm management strategies along with discontinuation of anthelmintic type.

SUMMARY

A study was aimed to observe the impact of withdrawal of anthelmintic type and increase in refugia through community dilution on reversion towards benzimidazole (BZ) susceptibility in *Haemonchus contortus* at an organized sheep farm in semi-arid Rajasthan. In sheep flocks of ICAR-Central Sheep and Wool Research Institute, Avikanagar reduced efficacy (80%) on faecal egg count reduction test (FECRT) with ED₅₀ of 0.239 µg thiabendazole (TBZ)/ml on egg hatch assay (EHA) indicative of emergence of *H. contortus* resistant to BZ was observed in the year 1995. Following discontinuation of BZ for deworming at farm since 1996, the efficacy varied between 0–44% in the year 2000 with predominance of BZ-resistant alleles (>90%) between the year 2005 to 2010.

The efficacy was still low (26% on FECRT, ED₅₀ of 0.196 µg TBZ/ml on EHA) even after 15 yr post withdrawal (in 2010). Later on, the concept of community dilution and refugia were implemented for worm management. With these strategies, during 2018–20 (22 yr post withdrawal) a significant improvement (86–93%) in efficacy of BZ was noticed against *H. contortus*. Simultaneously, the ED₅₀ values ranged from 0.011 to 0.119 with an average of 0.075±0.008 µg TBZ/ml on EHA with prevalence of BZ susceptible alleles up to 48% on allele specific PCR assay. The study indicates the possibility of reversion to BZ susceptibility in *H. contortus* population in farm area with community dilution and refugia based worm management strategies.

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