

Correlation between status of benzimidazole resistance in *Haemonchus contortus* on bio and molecular assays

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ABSTRACT

In relation to benzimidazole resistance in *Haemonchus contortus*, the diversity in genotypes was established through allele specific-polymerase chain reaction in infective larvae (L₃) of sheep from different agroclimatic regions of Rajasthan. The results of molecular assay were correlated with results obtained on biological assay (faecal egg count reduction test and egg hatch assay). The study was conducted in 22 sheep flocks and a total of 691 L₃ was genotyped for benzimidazole resistance. The overall genotypic frequency for homozygous resistant (rr) larvae ranged from 72.22% (north-eastern region) to 96.87% (eastern region) in farm flocks and from 61.79% (north-eastern region) to 95.83% (eastern region) in field flocks. The results of correlation revealed that phenotypic expression of benzimidazole resistance on faecal egg count reduction test was evident with occurrence of >30% rr genotypes in population. Likewise presence of >30% r alleles in population were found to express the benzimidazole resistance on egg hatch assay. With decrease in faecal egg count reduction%, there was a linear increase in the frequency of 'rr' genotype in population with maximum of 'rr' genotype (96.58%) in flocks exhibiting <50% efficacy for benzimidazole on faecal egg count reduction test.

Key words: Anthelmintic resistance, Benzimidazole, AS-PCR, Egg hatch assay, Faecal egg count reduction test, *Haemonchus contortus*

The extensive and sole reliance on anthelmintics with their indiscriminate use have resulted in emergence of parasites, resistant to one or more of the widely used anthelmintics throughout world including India (Singh and Swarnkar 2008). Prichard *et al.* (1980) reported that resistance is present when a greater frequency of individuals within a population able to tolerate doses of compound than in a normal population of the same species and is heritable. It indicates that loss of sensitivity is genetically transmitted in worm populations that were previously sensitive to the same drug (Sangster and Gill 1999). In this process, over several generations, resistant gene accumulates to such an extent that number of worms within a population survives following treatment

(Kohler 2001). Usually bioassays like *in vivo* faecal egg count reduction test (FECRT) and *in vitro* egg hatch assay (EHA) are used to detect anthelmintic resistance but these assays were reported to have an inherent limitation of lower sensitivity, labour and time intensive (Lacey *et al.* 1990). With the advent of biotechnology and molecular assays, it has now become possible to detect anthelmintic resistance at an early stage. The present study was designed to observe the correlation between frequency of resistant alleles and status of benzimidazole resistance in sheep flocks.

MATERIALS AND METHODS

The study was conducted in 22 flocks (3 farm and 19 field flocks) situated in 3 different agroclimatic regions of Rajasthan. The status of benzimidazole (BZ) resistance was determined by both FECRT and EHA as per Coles *et al.* (1992).

Isolation of infective larvae of Haemonchus contortus: Pooled faecal samples from each flock were cultured at 27 °C for 5 days and infective third stage larvae (L₃) of *H. contortus* were harvested and washed with distilled water. An aliquot of 10µl was aspirated from larval suspension and distributed in the form of several minute droplets over a glass

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Table 1. Genotypic and allelic frequencies for benzimidazole resistance in infective *Haemonchus contortus* larvae on AS-PCR

Region	Flock	No. tested	Genotypic frequency (%)			Allelic frequency (%)	
			rr	rS	SS	r	S
Farm flocks							
Eastern	1	32	96.87	3.13	0.00	98.44	1.56
North -Eastern	1	54	72.22	24.07	3.70	84.26	15.74
Northern	1	24	79.17	20.83	0.00	89.58	10.42
Overall		110	80.91	17.27	1.82	89.55	10.45
Field flocks							
Eastern	1	27	100.00	0.00	0.00	1.00	0.00
	2	35	91.43	5.71	2.86	94.29	5.71
	3	31	93.55	6.45	0.00	96.77	3.23
	4	45	97.78	2.22	0.00	98.89	1.11
	5	29	93.10	6.90	0.00	96.55	3.45
	6	25	100.00	0.00	0.00	100.00	0.00
	Total	192	95.83	3.65	0.52	97.66	2.34
North-Eastern	1	35	88.57	5.71	5.71	91.43	8.57
	2	52	69.23	5.77	25.00	72.12	27.88
	3	56	73.21	5.36	21.43	75.89	24.11
	4	27	40.74	40.74	18.52	61.11	38.89
	5	34	58.82	35.29	5.88	76.47	23.53
	6	23	17.39	69.57	13.04	52.17	47.83
	7	19	47.37	36.84	15.79	65.79	34.21
Northern	Total	246	61.79	21.95	16.26	72.76	27.24
	1	23	100.00	0.00	0.00	100.00	0.00
	2	21	100.00	0.00	0.00	100.00	0.00
	3	29	95.55	3.45	0.00	98.28	1.72
	4	25	80.00	16.00	4.00	88.00	12.00
	5	24	70.83	29.17	0.00	85.42	14.58
	6	21	76.19	23.81	0.00	88.10	11.90
Overall		581	79.35	13.94	7.23	86.32	13.68

331) amplified a product of 266 and 267 bp, respectively with common reverse primer (TGG-312) in separate reactions (Fig. 1).

Genotypic frequency: On comparison of allele specific genotypic frequencies from different geographical regions of Rajasthan, a significant ($P<0.05$) variation among different genotypes was observed (Table 1). The overall prevalence of rr genotypes in *H. contortus* larvae varied nonsignificantly among farm (80.91%) and field flocks (79.35%) of Rajasthan. Among farm flocks, the proportion of 'rr' genotypes in population varied significantly ($P<0.05$) from a minimum of 72.22% (north-eastern region) to a maximum of 96.87% (eastern region). The frequency of BZ susceptible genotypes (both SS and rS) was significantly ($P<0.001$) lower in eastern region ($<5.0\%$) compared to other regions ($>20\%$). The scenario of genotypic analysis for BZ resistance in infective larvae of *H. contortus* from field flocks revealed that among regions, frequency of 'rr' individuals ranged from 61.79% (North-eastern region) to 95.83% (Eastern region). Within region, flock-to-flock variation in frequency of 'rr' genotypes was not much in eastern region (91.43 to 100%) however

distinct variation was observed field flocks of North-eastern (17.39 to 88.57%) and Northern regions (70.83 to 100%). On comparison between benzimidazole efficacy and frequency of 'rr' genotype, an inverse linear relationship was observed (Fig. 2).

Allelic frequency: The analysis of allelic frequency on AS-PCR also exhibited similar trends to genotypic frequency in all the 3 geographical regions (Table 1). The overall proportion of BZ resistant (r) allele was significantly ($P<0.001$) higher (86.32–89.55%) as compared to only 10.45 to 13.68% prevalence of BZ susceptible (S) allele in infective larvae of *H. contortus*. Within flock, the frequency of r allele varied from 94.29 to 100% in eastern region, from 52.17 to 91.43% in north-eastern region and from 85.42 to 100% in Northern region.

Interaction for prevalence of benzimidazole resistance on FECRT, EHA and AS-PCR: On overall comparison between benzimidazole efficacy and frequency of 'rr' genotype, it was observed that population of larvae harvested from flocks having $>90\%$ reduction in the faecal egg counts for benzimidazole, comprised of 63.01% 'rr' genotypes

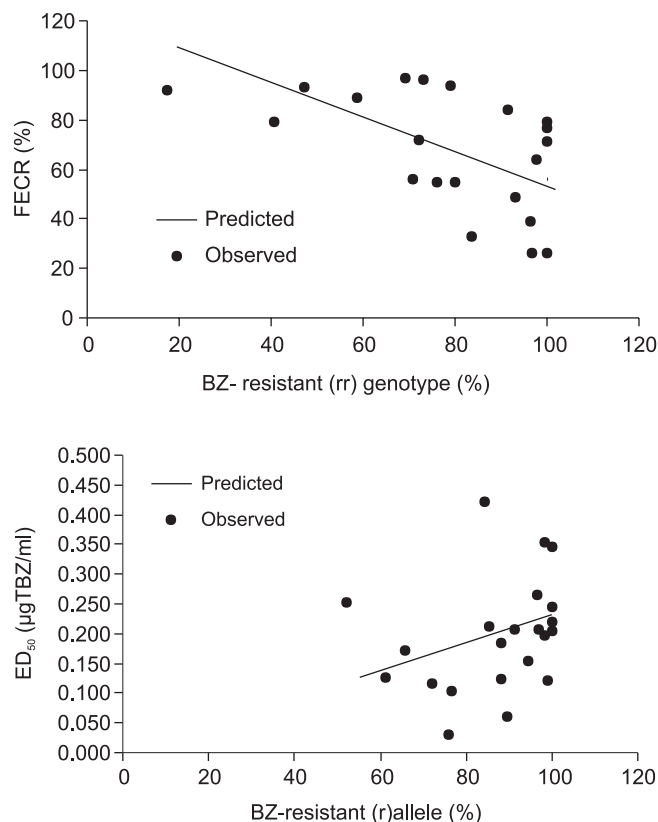


Fig. 2. Comparison between predicted and observed FECR and ED_{50} for benzimidazole against *Haemonchus contortus*.

(Table 2). With decrease in FECR%, there was a linear increase in the frequency of 'rr' genotype in population with maximum of 'rr' genotype (96.58%) in flocks exhibiting <50% efficacy for benzimidazole on FECRT. The similar type of distinct pattern was not evident between ED_{50} value and frequency of 'rr' genotypes (Fig. 2). The regression equations derived for interactions between %FECR or ED_{50} value and rr genotypes or alleles are as follows:

$$\begin{aligned} \%FECR &= 112.203 - 0.563 (rr\%), & r^2 &= 0.295 - (i) \\ ED_{50} &= 0.0391 + 0.001811 (r\%), & r^2 &= 0.073 - (ii) \end{aligned}$$

The results of correlation revealed that phenotypic expression of BZ resistance on FECRT was evident with

Table 2. Interaction between magnitude of benzimidazole-resistant genotype (rr) in infective larvae and *in vivo* efficacy of BZ against *Haemonchus contortus* in sheep

FECRT		AS-PCR	
FECR (%) range	No. of flocks examined	rr genotype (%)	No. rr / No. examined
> 90.0	5	63.01	109/173
70.0 – 90.0	6	75.26	146/194
50.0 – 70.0	4	84.35	97/115
< 50.0	4	96.58	113/117

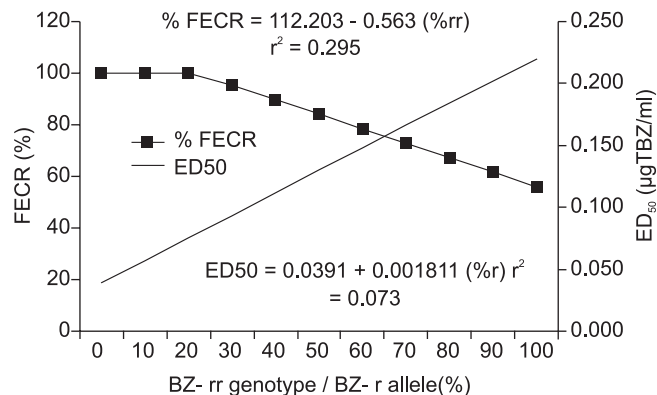


Fig. 3. Correlation between % FECR and frequency of rr genotype and between ED_{50} values and frequency of r allele for benzimidazole.

occurrence of >30% rr genotypes in population. Likewise presence of >30% r alleles in population were found to express the BZ resistance on EHA (Fig. 3). FECRT and EHA are relatively less sensitive as they detect resistance only when more than 25% of the present generation become resistant (Martin *et al.* 1989). In contrast, knowledge of molecular basis of BZ resistance has allowed the development of PCR based sensitive methods for early detection of genetic changes associated with selection of BZ resistance have the capacity to detect as little as 1% resistant individuals in a sample of a susceptible population and enables selective amplification from complex genomes (Gasser 2006). To overcome limitations of FECRT and EHA, molecular techniques are being employed for the diagnosis of anthelmintic resistance (Tiwari *et al.* 2006, 2007).

In the present study region based variation in prevalence of BZ resistance were observed with low prevalence in north-eastern region which would be attributed to low drench frequency (2 / year) in this region compared to other regions (>3 / year). Lower efficacy (<60%) of BZ in field flocks of eastern and northern regions compared to 91% efficacy in field flocks of North-eastern region could be due to the fact that in eastern and northern regions, drenching of flocks during extreme summer (June) is common practice (Anon 2002) when there is no *refugia*. Under these circumstances, it is expected that the eggs only from resistant worms reside in the body of host and contribute to genetic pool in next monsoon season which were available to sheep with likely decreased efficacy of anthelmintics during monsoon (Swarnkar *et al.* 2004). Regional differences in the susceptibility of parasites to chemotherapy suggested that gene frequency for resistant worms can differ from area to area before a drug is used (Coles 2005). The findings of the BZ resistance at the farms are in conformity with those reported earlier on eastern region (Singh *et al.* 1995, Swarnkar *et al.* 2001), North-eastern region (Swarnkar *et al.* 1999b) and on Northern region (Swarnkar *et al.* 1999a). Similar trends of high BZ resistance were recorded in earlier

studies also from field flocks (Anon 2002, Swarnkar *et al.* 2004).

The genotypic and allelic frequencies for BZ resistance was significantly higher (>80%) in both farm and field flocks. It could be due to the fact that prior to this study, the *H. contortus* populations of the farm and field flocks were all under selection pressure due to repeated use of BZ anthelmintic for worm control. As revealed on FECRT and EHA, a significant phenotypic resistance was present in all sheep flocks even before the treatment was performed during this study. So a higher proportion of homozygous rr individuals would not be unexpected in a situation like this (Elard *et al.* 1999). The lowest frequencies of 'rr' genotype was encountered in the populations of infective larvae from north-eastern region (particularly in flock 4 to 7) and it could be due to fact that from these flocks, larvae were collected during end of monsoon compared to during early monsoon in rest of the flocks. These findings support the hypothesis proposed by van Wyk (2001) that *refugia* play a significant role in the development of anthelmintic resistance. A moderate rise in proportion of susceptible genotype in *refugia* during late monsoon was reported to increase the efficacy of BZ compounds against gastrointestinal nematodes (Swarnkar *et al.* 2006). Similarly, from Rajasthan Tiwari *et al.* (2006) observed occurrence of 68% and 77% frequency for rr genotype and r allele respectively, in population of infective larvae of *H. contortus* using restriction fragment length polymorphism-polymerase chain reaction. Further, using adult male of *H. contortus*, Tiwari *et al.* (2007) observed the overall prevalence of BZ resistant allele to the tune of 87% with higher prevalence (93% and 98%) of BZ resistant genotypes in semi-arid (eastern) compared to 50% in arid (Northern) regions. Higher frequency of heterozygote individuals in BZ susceptible population would result in the rapid build up of a BZ resistant population, if the factors which allow the selection of resistant worms are persisting. Further Beech *et al.* (1994) reported that if the changes in the frequency have occurred solely as a result of genetic drift, caused by a reduction in effective population size during the selection process, the most abundant allele would be expected to approach fixation.

The analysis of data of % FECR and rr genotypes indicated an inverse relationship between them. Alvarez-Sanchez *et al.* (2005) reported a good correlation between FECRT and EHA data with the quantitative real time PCR results for the BZ susceptible and resistant strains of *H. contortus* and *Teladorsagia circumcincta* populations. In the present study, a moderate correlation was observed between % FECR and frequency of rr genotypes ($r=0.543$) and between ED₅₀ values and frequency of r allele ($r=0.270$). Besides this, the results of correlation revealed that phenotypic expression of BZ resistance as expressed on FECRT and EHA was evident with occurrence of >30% rr genotype in populations (Martin *et al.* 1989).

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