



Prevalence of benzimidazole resistant β - tubulin alleles in *Haemonchus contortus* larvae from sheep of Rajasthan

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ABSTRACT

The study was conducted to find out the feasibility of community dilution strategy in worm population for reversion to susceptibility against benzimidazole (BZ) anthelmintics. The prevalence of BZ-resistant β - tubulin alleles was determined through allele specific polymerase chain reaction (AS-PCR) in 1216 infective larvae of *Haemonchus contortus* isolated from sheep flocks of different origins. Both management of flocks and agro-climate has significant influence on the prevalence of homozygous BZ-resistant (rr) genotypes. The overall frequency of rr genotypes was relatively low in farm flocks and in arid Rajasthan. The maximum prevalence of BZ-susceptible was observed during winter (51.00%) and monsoon (24.59%) in arid and semi-arid farm. In field flocks, there was a moderate rise in proportion of BZ-susceptible genotypes during monsoon. The study exhibited that community dilution strategy for reversion of susceptibility to BZ anthelmintics in *H. contortus* could be feasible in farm conditions. The period from September to November in semi-arid and from June to February in arid agroclimatic conditions seem to be appropriate in increasing the frequency of BZ-susceptible alleles in the *refugia*.

Key words: Anthelmintic resistance, Benzimidazole, β -tubulin, *Haemonchus contortus*, *Refugia*, Sheep

Gastrointestinal (GI) nematodosis predominated by infection with *Haemonchus contortus* is world-wide responsible for sub-optimal productivity and unthriftiness in grazing animals. The control of parasites are mainly achieved by the use of anthelmintics however, the overdependence and/or misuse of anthelmintics have led to the emergence of anthelmintic resistance (Singh *et al.* 2001, Hosking and Kaminsky 2007). Despite the operationalization of information/awareness campaign to educate farmers, veterinarians and extension workers about sustainable methods to preserve the efficacy of the major anthelmintics (Waller 2003), the resistance problem seems to have escalated (Singh and Swarnkar 2009). Anthelmintic resistance is now accepted as a pre-adaptive phenomenon, and the genes or alleles conferring resistance to anthelmintics are believed to be in existence in unselected worm population. Consequently, for all anthelmintics that have been invented to date, it appears that the development of anthelmintic resistance is an inevitable consequence of their use but its development can be delayed. Worms that are in free-living sub-population (eggs and larval stages) are not exposed to the anthelmintics

and are said to be in *refugia* and are being viewed as an important tool to slow down the anthelmintic resistance. The aim of the present study was to ascertain the seasonal variation in genotypic frequencies for benzimidazole (BZ) resistance in *Haemonchus contortus* infective larvae and to find out the feasibility of community dilution strategy in worm population to decrease the incidence of BZ resistance in *H. contortus* and thus reversion to susceptibility for BZ anthelmintics in *H. contortus*.

MATERIALS AND METHODS

During the period from April 2005 to March 2006 faecal samples from ~ 20–30% of sheep in flocks, managed either at organized farms or in field in both arid and semi-arid agroclimatic regions of Rajasthan were collected at monthly interval. Pooled faecal samples on each occasion 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 10 μ l was aspirated from larval suspension and distributed in form of several minute droplets over a glass slide. To each droplet, equal amount of phosphate buffer saline (pH 7.2) was added and while monitoring under microscope, clean debris free individual *H. contortus* larvae was aspirated with 2 μ l volume using micropipette and transferred into PCR tube containing 3 μ l of PBS and stored at 4 °C till further use.

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A total of 1216 L₃ were genotyped to ascertain the variation in proportion of different genotypes in supra population with respect to BZ resistance through allele specific-polymerase chain reaction (AS-PCR) technique to identify the suitable time of intervention for dilution of BZ resistant worms through increasing refugia.

Extraction of genomic DNA: It was carried out as per Silvestre and Humbert (2000) with slight modification that exsheathment of larvae was not performed and the temperature of incubation for proteinase-K was also kept different. In each PCR tube, containing *H. contortus* larva, 2 µl proteinase-K (10 mg ml⁻¹) and 0.5 µl (10×) polymerase chain reaction buffer were added and centrifuged at 10 000 g for 3 min and kept at -20 °C for 1 h. Tubes were transferred to a thermal cycler and kept at 55°C for 90 min and at 95°C for 30 min to activate and deactivate the enzyme, respectively. The resultant DNA samples were stored at 4 °C for further use.

Primers: Following 2 sets of primers were used for amplification:

Common reverse primer (TGG 312): 5' – GGA ACC ATG TTC ACG GCT AAC – 3'

Susceptible primer (CAW 106): 5' – TAG AGA ACA CCG ATG AAA CAT T – 3'

Resistant primer (TGG 331): 5' – G TAG AGA ACA CCG ATG AAA CAT A – 3'

CAW 106 primer annealed with complimentary sequence of phenylalanine (TTC) codon, whereas TGG 331 primer annealed with complimentary sequence of tyrosine (TAC) at codon 200 of β-tubulin gene (Winterrowd *et al.* 2003). The PCR reaction mixture comprised 20 µl volume as 1X *Taq* polymerase buffer (2.0 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. Larvae that gave amplification only with susceptible primer were designated as homozygous susceptible (SS), the individuals that amplify only with resistant primer were designated as homozygous resistant (rr) and the individuals gave amplification with both the primers were designated as heterozygous (rS) for BZ resistance. Frequencies of different genotype and alleles were calculated as per Pierce (2003).

RESULTS AND DISCUSSION

AS-PCR discriminated resistant and susceptible populations based on the point mutation at codon 200 in β-tubulin isotype 1 gene. The susceptible allele specific primer (CAW-106) and resistant allele specific primer (TGG-331)

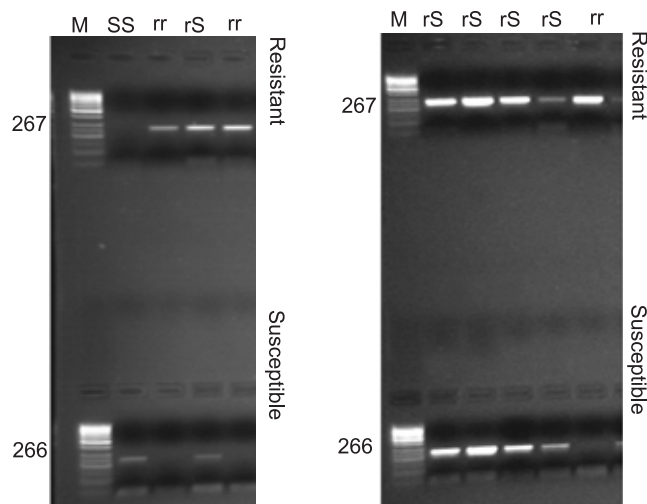


Fig. 1. AS-PCR profile of β-tubulin isotype gene 1 of *Haemonchus contortus* infective larvae (rr – homozygous resistant, rS, heterozygous susceptible; SS, homozygous susceptible; M, 50 bp ladder).

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

Genotypic frequency: The molecular characterization of β-tubulin isotype 1 gene from infective larvae of *H. contortus* in supra population of worms exhibited that overall genotypic frequencies with respect to BZ resistance were 84.04, 15.30 and 0.66% for homozygous resistant (rr), heterozygous susceptible (rS) and homozygous susceptible (SS) genotypes, respectively. The management of flocks had significant ($P < 0.05$) influence on the prevalence of genotypes with lower proportion (73.90%) of rr in worm population from farm flocks compared to those from field flocks (91.75%). Likewise agroclimatic conditions also exhibited significant ($P < 0.05$) effect on the prevalence of genotypes with higher proportion (91.01%) of rr genotypes in semi-arid agro-climate than arid agro-climate (76.99%) in Rajasthan (Table 1). The interaction of management and agro-climate revealed that though, there was relatively higher proportion of susceptible (rS and SS) genotypes in arid region under both the flock management system but the difference in proportion was significantly ($P < 0.05$) evident only in worm population from farm flocks. The seasonal analysis exhibited that frequency of susceptible genotypes in supra population at arid farm remained at the lowest (27.00%) during summer, which increased moderately to 30.39% during monsoon and significantly to 51.00% during winter. However, in semi-arid farm though frequency of susceptible genotypes increased significantly during monsoon compared to summer but declined thereafter to minimum during winter. In field flocks there was a moderate rise in proportion of BZ-susceptible genotypes during monsoon in both the agroclimatic regions. The monthly prevalence of rr genotypes remained minimum in October in all the conditions except

Table 1. Genotypic and allelic frequencies for benzimidazole resistance in infective *Haemonchus contortus* larvae from sheep flocks of Rajasthan

	Genotypic frequency (%)			Allelic frequency (%)	
	rr	rS	SS	r	S
Overall (1216)	84.04	15.3	0.66	91.69	8.31
Management					
Farm (525)	73.9	24.95	1.14	86.38	13.62
Field (691)	91.75	7.96	0.29	95.73	4.27
Agro-climate					
Arid (604)	76.99	22.02	0.99	88	12
Semi-arid (612)	91.01	8.66	0.33	95.34	4.66
Management $\times$ Agro-climate					
Arid farm (289)	63.67	34.6	1.73	80.97	19.03
Semi-arid farm (236)	86.44	13.14	0.42	93.01	6.99
Arid field (315)	89.21	10.48	0.32	94.44	5.56
Semi-arid field (376)	93.88	5.85	0.27	96.81	3.19

Figure in parentheses indicates number of larvae examined.

in arid farm where it was in January. The frequency of rr genotypes reached to 100.00% just after the period of climatic extremes (Fig. 2).

Allelic frequency: The analysis of allelic frequency on AS-PCR exhibited the overall prevalence of 91.69% for BZ-resistant allele with marginally lower frequency (86.38%)

in farm flocks compared to field flocks (95.93%). Similarly, the proportion of BZ-resistant alleles ranged from 88.00% in arid to 95.34% in semi-arid Rajasthan (Table 1). The interaction of management and agro-climate showed significantly lower frequency of BZ-resistant alleles (80.97%) only in worms from flocks of arid farm compared to other flocks (>90%). The seasonal analysis exhibited universal rise in frequency of BZ-susceptible allele during monsoon irrespective of agroclimatic conditions and flock management, however exceptionally there was further rise in frequency of BZ-susceptible allele during winter at arid farm (Fig. 2).

Knowledge of molecular basis of BZ resistance has allowed the development of PCR based sensitive methods to detect as little as 1% resistant individuals in a sample of a worm population and enables selective amplification from complex genomes (Gasser 2006). To overcome limitations of other bio-assays, molecular techniques are being employed for the diagnosis of anthelmintic resistance (Silvestre and Humbert 2002, Alvarez-Sánchez 2005, Tiwari *et al.* 2006, 2007). Rufener *et al.* (2009) developed an allele-specific PCR to monitor the prevalence of alanine (198) encoding alleles in the β -tubulin isotype 1 gene pool of *H. contortus* in the field. In any parasite ecosystem, it is only the parasitic sub-population (the parasites within the host) that can be exposed to any anthelmintic treatment. On the other hand, worms that

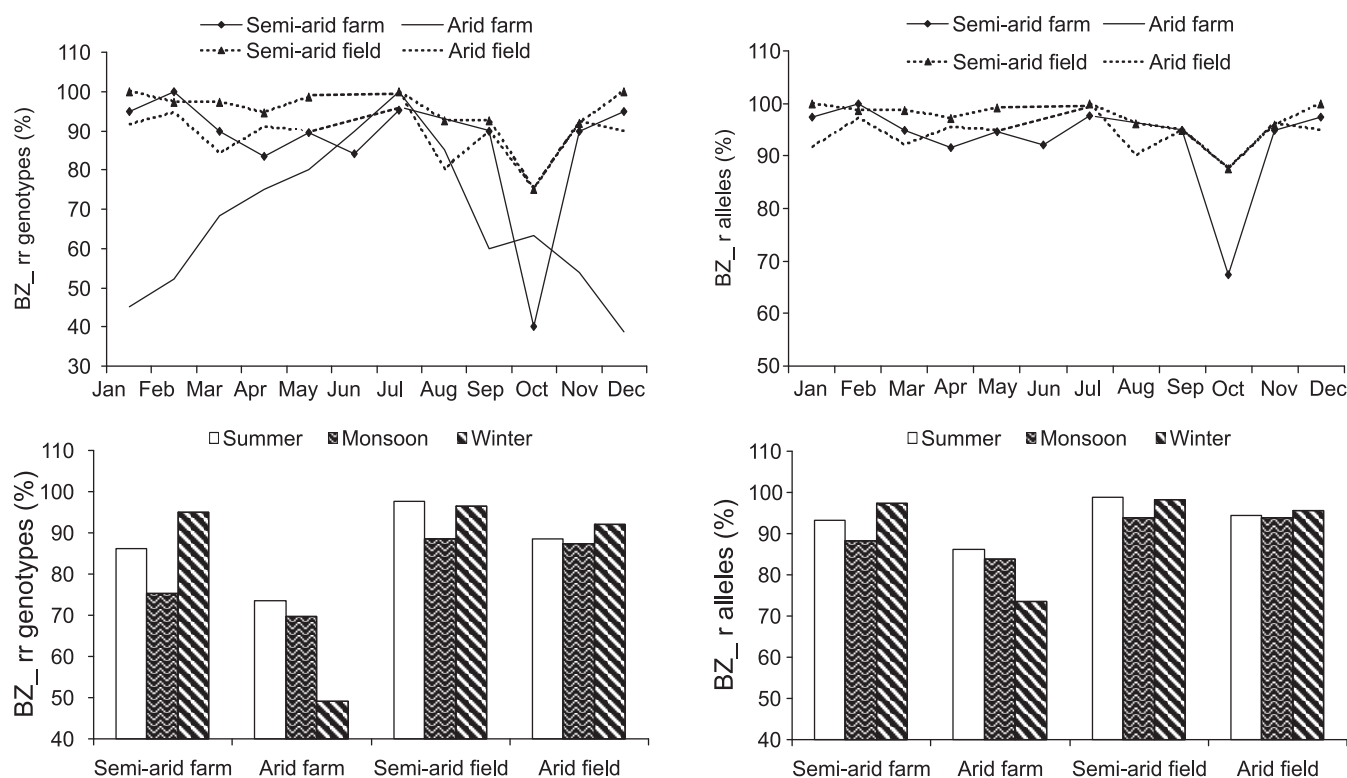


Fig. 2. Monthly and seasonal frequency (%) of BZ-resistant genotypes/alleles in infective *Haemonchus contortus* larvae from sheep flocks of Rajasthan.

are in free-living sub-population (eggs and larval stages) are not exposed to the anthelmintics and are said to be *in refugia*. Any worms in sheep that are not treated also contribute to *refugia*. One of the important factors influencing the rate at which resistance develops in a worm population is the relative size of the exposed population and the unexposed or *refugia* population. In general, the larger the *refugia* population in comparison to the exposed population, the more slowly the resistance will develop. Nielsen *et al.* (2007) reported that *refugia* were smallest during the winter in cold climate and during summer in hot and warm climate. Drenching of flocks during extreme summer (June) is common practice (Swarnkar *et al.* 2008) when there is no *refugia*. Under these circumstances, it appears that the eggs only from resistant worms (residing in host) will contribute to genetic pool in next monsoon and available to sheep flocks (Swarnkar *et al.* 2004, Singh *et al.* 2008). 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 genotypic and allelic frequencies for BZ resistance were significantly higher 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 under massive selection pressure due to repeated use of BZ anthelmintic for worm control. A higher proportion of homozygous rr individuals during climatic extremes 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 compared to 50% in arid regions. In North-West India, Garg and Yadav (2009), observed that in population of infective larvae from sheep had higher frequency of r alleles at Pantnagar (0.85) compared to Kedarkattha (0.70) and Bareilly (0.62) and concluded that management practices have a direct bearing on the spread of BZ resistant alleles. In Swedish sheep flocks, Höglund *et al.* (2009) used pyrosequencing assay for the analysis of BZ resistance targeting the P200 mutation in the parasite's β -tubulin gene and detected BZ resistant allele frequencies of >40% in the *Haemonchus*-positive farms and 100% resistant alleles in the clinically most resistant farms. 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 high prevalence of BZ resistant genotypes in worm population indicated that it is a serious problem in both organized and unorganized sheep flocks. Coles *et al.* (1995) stated that a high selection pressure by anthelmintic treatment was neither necessary nor sufficient to promote the anthelmintic resistance development in real life and Silvestre *et al.* (2002) hypothesized that anthelmintic resistance may develop under an efficient (treatments are performed at the right time) rather than under apparent (number of treatments) selection pressure. A selection pressure is efficient when the resistant worms, that survived the anthelmintic treatment, can contribute for a large part to the subsequent worm generation. In addition to the continuous indiscriminate use of anthelmintics since their availability in the market, the other possible reasons for the higher incidence of anthelmintic resistance in *H. contortus* include practice of under dosing due to incorrect dose calculation, faulty administration or use of spurious drugs (Smith *et al.* 1999) and use of anthelmintics when the size of *refugia* in population is almost nil (van Wyk 2001). Under situations of continuous drench and flock isolation, applications of anthelmintic treatments were found to enhance development of anthelmintic resistance in nematodes (Papadopoulos *et al.* 2001). Frequent migration of sheep flocks throughout the state as well as failure to use anthelmintic of same class in a region at a time leads to further dispersal of resistant worms in the entire region and this phenomenon is known as 'borrowing of resistance' (Hertzberg *et al.* 2000, Swarnkar *et al.* 2003).

Normally, in unselected population of worms, resistant (r) alleles are either absent or are present at very low frequencies and could be assumed that these alleles have a selective disadvantage for fitness. If anthelmintics are used and any r-alleles are present, they will increase in frequency. If anthelmintic use is discontinued, natural selection might be expected to reduce the prevalence of r-alleles in favour of the fitter, fully susceptible parasites and the population would, theoretically, revert towards full susceptibility. Reversion refers to a population drift back to drug sensitivity after the parasite population had become resistant to that particular drug (Leathwick *et al.* 2001). Supportive data on the occurrence of reversion in the field are limited. Hall *et al.* (1982) and Martin (1987) found no reversion in BZ resistant worms after 6–12 generations but Simpkin and Coles (1978) reported a reduction in BZ resistance if resistant strains were cycled without further exposure to BZ. Parasites in *refugia* are not exposed to selection for resistance and thereby provide a source of susceptible gene alleles to dilute r-alleles in the population (van Wyk 2001). Accordingly maintenance of adequate parasite *refugia* can slow down the development of anthelmintic resistance as was confirmed by experimental

studies (Dobson *et al.* 2001) as well as by computer modelling (Barnes *et al.* 1995, Smith *et al.* 1999). A success in reversion of parasites susceptibility was observed through community dilution approach by Bird *et al.* (2001) and anthelmintic efficacy in the control of nematode parasites of small ruminants was restored by exploiting *refugia* (Sissay *et al.* 2006). It is clear from present study that community dilution strategy for reversion of susceptibility to BZ in *H. contortus* could be feasible in farm condition. Under prevailing management and grazing practices in field flocks, the variation in genotypic and allelic frequency could not be observed. The period from September to November in semi-arid and from June to February in arid Rajasthan seems to be appropriate in increasing the frequency of BZ-susceptible alleles in the *refugia*.

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