



Detection of benzimidazole resistance in *Haemonchus contortus* populations collected from sheep of Uttarakhand using allele specific PCR assay

NAVEEN PANDEY¹ and STUTI VATSYA²

Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand 263145 India

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ABSTRACT

Allele specific PCR (AS-PCR) was standardized to detect genotypic and allelic frequencies of *Haemonchus contortus* worm populations of 2 agro-climatic regions viz. Tarai and Hill regions of Uttarakhand. Genotyping of *H. contortus* worms revealed that overall frequency of homozygous susceptible worms were significantly higher (0.61) than homozygous resistant (0.17) worms. Within Tarai region, the frequency of homozygous resistant worms were significantly higher (0.48) in Pantnagar than homozygous susceptible (0.20) worms whereas, significantly higher frequency of homozygous susceptible worms were found in Haldwani (0.52) and Tanakpur (0.80). In Hills, the frequency of homozygous susceptible worms was significantly higher than homozygous resistant worms collected from Nainital (0.72 and 0.08 respectively) and Almora (0.84 and 0.04 respectively).

Overall, the frequency of susceptible allele was significantly higher (0.72) than resistant allele (0.28). In Tarai region, frequency of resistant allele (0.36) was significantly lower than susceptible allele (0.64). The frequency of resistant allele was significantly higher in Pantnagar (0.64) whereas significantly higher frequency of susceptible allele was recorded in *H. contortus* collected from Haldwani (0.68) and Tanakpur (0.86). In Hill region, frequency of resistant allele (0.14) was significantly lower than susceptible allele (0.86).

Key words: Benzimidazole, *Haemonchus contortus*, Hill region, Resistance, Susceptible, Tarai region

Haemonchus contortus is a major cause of economic losses in small ruminants as it is responsible for retarded growth, weight loss, disorder in fertility, loss in milk production and even mortalities (Singh *et al.* 2011, Varady *et al.* 2011). At present, gastrointestinal (GI) parasites are controlled by anthelmintics. However, non judicious use of anthelmintics has contributed to rapid emergence of anthelmintic resistance in GI nematodes in India (Garg and Yadav 2009). Benzimidazole resistance develops in *H. contortus* in several ways like change in the molecular target (Gilleard 2006), change in drug transport (Smith and Prichard 2002) and change in drug metabolism (Cvilink *et al.* 2009). A mutation (phenylalanine to tyrosine) at amino acid 200 of β -tubulin isotype-1 gene is responsible for causing resistance (Elard and Humbert 1999). Beside this, similar mutation at 167 amino acid of β -tubulin isotype-1 (Prichard 2001) and another mutation (Glutamate to Alanine) at 198 amino acid (Ghisi *et al.* 2007) were also found causing benzimidazole resistance.

Molecular methods are highly sensitive and specific (Elard

and Humbert 1999, Silvestre and Humbert 2000, Alvarez-Sanchez *et al.* 2005, Coles 2006, Tiwari *et al.* 2006, Tiwari *et al.* 2007, Ghisi *et al.* 2007, Garg and Yadav 2009) having ability for early detection of benzimidazole resistance within worm population (Roos *et al.* 1995) as compared to *in vivo* and *in vitro* tests. The present study was planned to determine benzimidazole resistance in *H. contortus* populations collected from different agro-climatic zones of Uttarakhand using allele-specific PCR assay.

MATERIALS AND METHODS

Collection of parasites: Adult male *H. contortus* were collected from the abomasii of sheep slaughtered at abattoirs located in 2 different agro-climatic regions of Uttarakhand viz. the Tarai (Pantnagar- a sub Himalayan region having sub-tropical climate, Haldwani and Tanakpur -both sub Himalayan regions having sub-tropical climate) and Hill {(Nainital 1939 m) and (Almora 2412 m) above sea level— both high altitude temperate regions)}. The adult male worms were washed properly and kept separately at -20°C in separate tubes till used for DNA isolation.

Genomic DNA isolation: Genomic DNA was isolated from 125 worms (25 each from Pantnagar, Haldwani, Tanakpur, Nainital and Almora) on individual basis as per Anonymous

Present address: ¹ M.V.Sc. Scholar (nnp.pandey08@gmail.com),
²Associate Professor, (stutivatsya@gmail.com), Department of Parasitology, College of Veterinary and Animal Sciences.

(1999) with modifications. Briefly, the worms were thoroughly washed in distilled water. Individual worm was collected and suspended in 500 µl of worm lysis solution (100 mM Tris-Cl, 100 mM NaCl, 50 mM EDTA, 1% SDS, 1% (β-mercaptoethanol, 100 µg/ml proteinase K and 20 µg/ml RNase A) in separate tubes and frozen at -60°C for 1 h. The tubes were thawed and incubated at 65°C for 1 h with occasional agitation. After incubation, mixture of phenol: chloroform: isoamyl-alcohol (25:24:1) 500 µl was added to each tube. The contents of the tubes were mixed by inverting the tubes gently till a homogenous white solution was visible. The tubes were centrifuged at 10 000 rpm for 10 min at 4°C. From centrifuged tube, the aqueous phase was carefully collected in a separate tube taking care that the interface was avoided and then equal volumes of chloroform: isoamyl-alcohol (24:1) was added. The contents of the tubes were again mixed and centrifuged at 10 000 rpm for 10 min at 4°C.

The aqueous phase thus formed in each tube was again collected in a separate tube and 500 µl of chilled absolute ethanol and 100 µl of 5 M sodium acetate were added in each tube. The contents of each tube were mixed and then all tubes were kept at -20°C for 1 h followed by centrifugation at 12 000 rpm for 15 min. The resulting supernatant in each tube was discarded and the DNA pellet settled at bottom of tubes was mixed with 70% ethanol. It was again centrifuged at 10 000 rpm for 3–4 min at 4°C and the supernatant was discarded. The washed DNA pellet was suspended in 30 µl of TE buffer (10 mM Tris-Cl and 1 mM EDTA, pH 8.0) and kept at -20°C till further use.

Genotyping of adult *H. contortus*: For genotyping, initially a 840 bp isotype 1 β-tubulin gene was amplified and then an allele specific PCR was standardized using 3 primer technique (Silvester and Humbert 2000). Allele specific PCR was carried out with 25 µl reaction mixture containing 2 non-specific primers (forward and reverse) and either allele specific (resistance or susceptible) primer. The products of allele-specific PCR were analyzed by gel electrophoresis using 1.5% agarose. On gel electrophoresis, a resistant

specific band of 250 bp, susceptible specific band of 550 bp and a 650 bp non specific band were observed. Based on electrophoretic profile of PCR products, the adult worms were genotyped as homozygous resistant (rr), homozygous susceptible (SS) and heterozygous (rS).

Statistical analysis: Chi-square test (Snedecor and Cochran 1994) was used to compare the genotypic and allelic frequencies of adult worms collected from different agro-climatic regions of Uttarakhand.

RESULTS AND DISCUSSION

The overall frequency of homozygous resistant (rr) (0.17) was significantly ($P < 0.005$) lower than homozygous susceptible (SS) (0.61) individuals in the 2 agro-climatic zones, viz. Tarai and Hill of Uttarakhand. The frequency of resistant individual *H. contortus* was significantly ($P < 0.005$) higher in Tarai region (0.24 rr and 0.25 rS) than at Hill region (0.06 rr and 0.16 rS) (Table 1).

Within Tarai region, the frequency of resistant individuals at Pantnagar was (0.48 rr and 0.32 rS) significantly higher ($P < 0.005$) followed by Haldwani (0.16 rr and 0.32 rS) and Tanakpur (0.08 rr and 0.12 rS). In Hill region, the frequency of resistant individual in Nainital was higher (0.08 rr and 0.20 rS) followed by Almora (0.04 rr and 0.12 rS).

Overall, the prevalence of benzimidazole susceptible allele (S) was significantly ($P < 0.005$) higher (72%) as compared to resistant allele (r) (28%). Similar trends were observed in Tarai region (r=36%, S=64%) and Hill (r=14% and S=86%) region. However, the frequency of resistant and susceptible alleles varied significantly between places at Tarai region (Pantnagar 0.64, Haldwani 0.32 and Tanakpur 0.14) and Hill region (Nainital 0.18 and Almora 0.10).

The AS-PCR standardized in this study is a rapid and convenient means of genetic characterization of worms' population for benzimidazole resistance and susceptibility in *H. contortus*. One of the biggest advantages of this technique is that it can detect early emergence of first mutant worm in a worm population (Elard *et al.* 1998). There was prevalence of only 8 and 4% homozygous resistant worms in Nainital and

Table 1. Genotypic and allelic frequencies of *Haemonchus contortus* of different agro-climatic regions of Uttarakhand

Location of abattoir		No. of worms screened	Genotype of worms			Allelic frequency	
			Homozygous resistant (rr)	Homozygous susceptible (SS)	Heterozygous (rS)	Resistant (r)	Susceptible (S)
Tarai	Pantnagar	25	12* (0.48)	5* (0.20)	8* (0.32)	0.64*	0.36*
	Haldwani	25	4* (0.16)	13* (0.52)	8* (0.32)	0.32*	0.68*
	Tanakpur	25	2* (0.08)	20* (0.80)	3* (0.12)	0.14*	0.86*
Total		75	18* (0.24)	38* (0.51)	19* (0.25)	0.36*	0.64*
Hill	Nainital	25	2* (0.08)	18* (0.72)	5* (0.20)	0.18*	0.82*
	Almora	25	1* (0.04)	21* (0.84)	3* (0.12)	0.10*	0.90*
Total		50	3 (0.06)	39 (0.78)	8 (0.16)	0.14	0.86
Overall total		125	21* (0.17)	77* (0.61)	27* (0.22)	0.28*	0.72*

* indicates significance at $P < 0.005$. Values in parenthesis indicate percentage.

Almora, respectively. Also the frequency of resistant alleles was 0.18 in Nainital and 0.10 in Almora. These are high altitude areas with extremely cold climatic conditions detrimental for survival of infective stages of *H. contortus*, causing low infection and hence lesser use of anthelmintics by livestock owners. Also the livestock owners in these areas feed their animals indigenous herbs with anthelmintic properties instead of oral administration of anthelmintics. Our results are in consonance with those of Garg and Yadav (2009) who reported higher frequencies of benzimidazole resistant (r) alleles (0.57) in Pantnagar of Tarai region as compared to Mukteswar (0.08) of Hill region of Uttarakhand. They also recorded higher frequencies of resistant alleles following genotyping of infective larvae of *H. contortus* from intensively managed sheep farms at Pantnagar (0.85) in comparison to Kedarkattha (0.70), yet another high altitude region of Hills of Uttarakhand.

In and around Pantnagar of Tarai region, livestock owners drench their animals frequently (3–4 times a year) and indiscriminately with benzimidazoles attributing to higher prevalence of resistant alleles (Garg and Yadav 2009).

Our results indicated that the benzimidazoles resistance has begun to emerge even in the higher altitude areas of Uttarakhand. The presence of benzimidazole resistance in *H. contortus* population of Hill region may be due to the fact that during extreme cold climate the animals are migrated to the near by Tarai areas. During migration, the animal may pick up resistant infective stages (L_3) already present in these areas. As the temperature in Hills began to increase, the animals are migrated back to high altitude areas. Thus aiding in spread of resistant alleles in these areas also, as it is evident from the genotyping results.

The presence of 24 and 17% homozygous resistant (rr) *H. contortus* in Tarai and Hill region, respectively, may indicate a large population of *Haemonchus* to be phenotypically susceptible to benzimidazoles, however, their indiscriminate and non-judicious use may result in rapid development of benzimidazole resistance in future. Moreover, the AS-PCR employed in the present investigation can't detect mutations other than F200Y in β -tubulin gene. If other mutations F167 (Prichard 2001) and E198A (Ghisi *et al.* 2007) also exist in worm populations under study, then the level of resistance could be much higher.

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