



Molecular detection of benzimidazole resistance in *Haemonchus contortus* of sheep in Punjab

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ABSTRACT

Single nucleotide polymorphism (SNP) at codon 167, 198 and 200 of β -tubulin isotype 1 gene accounts for benzimidazole resistance in *Haemonchus contortus*. To assess the anthelmintic resistance to benzimidazole group, allele specific polymerase chain reaction (AS-PCR) was employed on 50 adults of local abattoir of Ludhiana and 138 harvested larvae of *H. contortus* from faecal samples collected from field flocks of 6 districts of western zone of Punjab. The adults were found to be homozygous resistant (rr) showing 100% prevalence of resistant allele (r). AS-PCR on 138 larvae collected from different districts of Punjab showed 49.28% (69) homozygous resistant (rr), 3.62% (5) homozygous susceptible (SS) and 46.37% (64) heterozygous (rS) population. Species was confirmed as *H. contortus* by PCR-RFLP from the three fragments of size 462 bp, 211 bp and 147 bp. The pioneer study on the molecular diagnosis of benzimidazole resistance in *H. contortus* showed an overall allele frequency for resistant and susceptible population to be 0.72 (TTC) and 0.28 (TAC), respectively indicating development of high level of resistance in sheep.

Key words: Anthelmintic resistance, AS-PCR, Benzimidazole, *Haemonchus contortus*, Punjab, Sheep

Gastrointestinal parasitism is the one of the major obstacle in the production of small ruminant industry due to the production losses incurred by various helminthic parasites. High prevalence of these infections has been observed in Punjab including western zone (Singla 1995, Singh *et al.* 2017). Beside various helminthic parasites, major impetus on the small ruminants especially of sheep farming is caused by *Haemonchus contortus* (Santos *et al.* 2012). *Haemonchus contortus* is a predominant parasite having ubiquitous prevalence in the small ruminants and is of considerable socio-economic concern because of high blood sucking capacity of 0.05 ml/worm daily. The estimated treatment cost due to *H. contortus* is around \$103 million in India (McLeod 2004). Till date the control of this haemtophagus parasite relies upon the chemotherapy. But non-judicious use and under dosing of drug, self deworming without veterinary assistance, are the leading factors responsible for the emergence of anthelmintic resistance. Beside the high biotic potential and short pre-

patent period, it showed its ability to develop resistance against almost all major classes of anthelmintics rapidly (Kotze and Prichard 2016) and turned out to be a suitable model for studying resistance besides *Caenorhabditis elegans* as *H. contortus* is phylogenetically closest to *C. elegans* (clade V) parasites. In Punjab, small ruminants are reared mainly by the marginal and landless farmers who prioritize the use of broad spectrum anthelmintics especially benzimidazoles (BZ) to combat the desolation caused by barber pole worm (Singh *et al.* 2017). As a consequence, the frequent and indiscriminate use of BZ has paved a pathway for inception of anthelmintic resistance (AR), thus increasing drug pressure for the selection of resistant alleles (Stuchlikova *et al.* 2018).

Anthelmintic resistance has become a serious problem in veterinary field, so a number of tests have been devised for the detection of BZ resistance namely *in vivo*, *in vitro* and molecular assays (Coles *et al.* 2006). Faecal egg count reduction test (FECRT) is a gold standard *in vivo* test but is labour and cost intensive. Egg Hatch Test (EHT) being the most commonly employed *in vitro* phenotypic test but its main drawback is requirement of fresh faecal sample as prevention of egg hatching is the main determinant for resistance diagnosis. Apart from EHT, is another *in vitro* technique of larval development assay (LDA), which has its own lacunae of being labour intensive and requires a week to give the results. Molecular tests include conventional PCR (Silvestre and Humbert 2000), real-time

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PCR (Alvarez-Sanchez *et al.* 2005) and pyrosequencing (Demeler *et al.* 2013). Conventional techniques (*in vivo* and *in vitro*) are considered to be less sensitive in comparison to molecular tests, where the former can detect resistance in isolates with at least 25% resistant individuals, while the latter can detect up to 1% resistant individuals in the population (Pape *et al.* 2003).

Studies on BZ resistance detection have thrown light on the role of Single Nucleotide Polymorphisms (SNPs) on the β -tubulin isotype 1 gene at positions 167, 198 and 200 (Barrere *et al.* 2013). In India, resistance studies have elucidated the importance of substitution F200Y (replacement of phenylalanine by tyrosine), however, a mutation at position 198 from a single origin in *H. contortus* has been reported from southern India (Chaudhry *et al.* 2015). In the current study, status of resistance in *H. contortus* was determined in western zone of Punjab employing molecular techniques to elucidate the type of SNP associated with BZ class of anthelmintics.

MATERIALS AND METHODS

Sample collection and study area: Adults of *H. contortus* were collected from the abomasia of 10 sheep from local abattoir of Ludhiana district. On the basis of morphological characters described in Soulsby (1982), males and females were separated and thoroughly washed in PBS before being stored in separate tubes at 4°C till further use for DNA isolation.

Faecal samples were collected per-rectally from extensively managed sheep flocks from western zone of Punjab state comprising of 6 districts, viz. Barnala, Bathinda, Mansa, Moga, Muktsar and Sangrur. The faecal samples were transported on ice to laboratory to prevent parasites development. The qualitative analysis of all samples for the presence of strongyle type eggs followed by quantitative faecal egg count based on modified McMaster technique to denote the parasitic load in eggs per gram was done. The samples from each farm of the respective districts with high EPG were pooled and subjected to coproculture technique at room temperature with adequate moisture. Thereafter, the third stage larvae of *H. contortus* were collected, thoroughly washed and stored at 4°C till use for DNA isolation.

Genomic DNA extraction: The genomic DNA of a total of 50 adult *H. contortus* (25 whole adult males and 25 adult females excluding uterus) were isolated individually using standard protocol (Beech *et al.* 1994) after modifications. Worms were thoroughly washed with distilled water and then suspended individually in 224.2 μ l of digestion mixture comprising 200 μ l STE (1 M sodium chloride, 0.5 M Tris-HCl, 0.5 M EDTA), 10 μ l of 10% SDS, 10 μ l β mercaptoethanol, 4 μ l Proteinase-K (10 mg/ml), 0.2 μ l RNase A (100 mg/ml) and incubated at 56°C for 4h. To the digested suspension, 200 μ l of phenol: chloroform: *iso*-amyl alcohol (25:24:1) mixture was added and centrifuged at 12,000 rpm for 5 min at room temperature. The aqueous phase was collected in a new tube and 270 μ l of absolute

iso-propanol and 130 μ l of 7.5 M ammonium acetate were added and centrifuged at 14,000 rpm for 20 min at room temperature. The supernatant was discarded and the pellet was washed with 100 μ l of 70% ethanol and centrifuged at 14,000 rpm for 10 min. The supernatant was discarded and the pellet was kept in an open tube until the evaporation of visible traces of ethanol. Then the DNA pellet was dissolved in 40 ml of 1X TE buffer (pH 8.0) and stored at -20°C till further use.

For DNA isolation, L₃ larvae which were alive and showing good motility were utilized. DNA was isolated from individual third stage larva by using the method described by Silvestre and Humbert (2000) with slight modifications. Ex-sheathment of larvae was done by incubating 4 ml of larval suspension in distilled water (less than 1,000 larvae/ml) and 180 μ l of 4% sodium hypochlorite solution for 20 min in a petridish. After ex-sheathment larvae were washed twice with distilled water and re-suspended in double distilled water. Single ex-sheathed larva was carefully removed with 2 μ l of suspension using a micropipette under microscopic surveillance and placed in a PCR tube. DNA was extracted by adding 5 μ l of extraction buffer [1 mM tris-HCl, 0.1 mM EDTA, 5 mg/ml Proteinase K and 0.5% NP-40 buffer], freeze (-20°C) and thawed (37°C) three times and incubating tubes at 56°C for 2h in a thermal cycler followed by proteinase K inactivation at 95°C for 20 min also in thermal cycler. The tubes were stored at -20°C until molecular analysis.

Amplification of β -tubulin gene: The method used was described by Silvestre and Humbert (2000) with minor modifications.

Primary and nested PCR: The amplification of the β -tubulin gene was conducted by primary PCR with PCR mixture consisting of 50 ng (1 μ l) of digested adult, 5 $\times$ Green GoTaq Flexi Buffer (Promega), 1.5 mM of 25 mM MgCl₂ (Promega), 200 μ M of 10 mM dNTPs mix (Thermo Scientific), 10 pmol of each primer Pn1 (5' GGC AAA TAT GTC CCA CGT GC 3') and Pn2 (5' GAA GCG CGA TAC GCT TGA GC 3'), 1.0 U of Taq DNA Polymerase. The PCR was performed with initial denaturation at 95°C for 5 min followed by denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 60 s for 38 cycles and then a final extension at 72°C for 10 min. However, primary PCR employed on *H. contortus* larvae used 5 μ l of template DNA with same cyclic conditions but only 20 cycles of PCR were run in comparison to 38 cycles on adult samples. The PCR amplicons were checked for amplification by electrophoresis on a 1% agarose gel in TBE (1 $\times$) buffer at 120 V for 1h. The DNA migration and resolution pattern was examined by UV transillumination [Syngene Gel documentation system (UK)] technique and the picture was documented by photography.

The product obtained after primary PCR was diluted 1:20 times with nuclease free water and then used as template for nested PCR. The PCR mixture consisted of 1 μ l of template (primary PCR product), 5 $\times$ Green GoTaq Flexi Buffer, 1.5 mM of 25 mM MgCl₂, 200 μ M of 10 mM dNTPs

mix, 10 pmol of each primer Pn3 (5' GTG CTG TTC TTG TTG ATC TC 3') and Pn4 (5' GAT CAG CAT TCA GCT GTC CA 3'), 1.0 U of Taq DNA Polymerase. The PCR was run on a thermocycler with initial denaturation at 95°C for 5 min then denaturation at 95°C for 30 s, primer annealing at 57°C for 30 s and strand extension at 72°C for 60 s for 35 cycles and a final extension at 72°C for 10 min. Same conditions were employed on the primary PCR product of adult and larvae of *H. contortus*. The PCR amplicons were checked for amplification by electrophoresis on a 1.5% agarose gel in TBE (1×) buffer at 120 V for 1h. The DNA migration and resolution pattern was examined by UV transillumination [Syngene Gel documentation system (UK)] technique and the picture was documented by photography.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP): 12 µl of the amplified nested product was digested with the restriction enzyme *RsaI* for species identification. The digestion process was performed for 1 h at 37°C in incubator and the resulting fragments were separated by electrophoresis on a 2.0% agarose gel. Their lengths were used for species identification. The reaction composition of PCR-RFLP was: 12 µl amplified nested product, 2 µl of 10× buffer, 1 µl of *RsaI* enzyme, 5 µl of NFW, thus making a total volume of 20 µl. PCR-RFLP was employed only on the larval DNA.

Allele Specific PCR for detection of benzimidazole resistance: AS-PCR reaction was performed using nested PCR product as template. Two aliquots (1 µl) of the (Pn3-Pn4) nested product were used to determine the resistance of adult parasites against BZ group of drugs. The system was divided into two mixes, each containing two non-specific primers [Ph1 (5' GGA ACG ATG GAC TCC TTT CG 3') and Ph2 (5' GGG AAT CGA AGG CAG GTC GT 3')] and one allele-specific primer. 1 µl of nested product used as a template for amplification of AS-PCR with 10 pmol of Ph1 and Ph2, 20 pmol of Ph3 resistant allele primer (5' CTG GTA GAG AAC ACC GAT GAA ACA TA 3') in one mix and 20 pmol of Ph4 susceptible allele primer (5' ATA CAG AGC TTC GTT GTC AAT ACA GA 3') in another mix, 5× Green GoTaq Flexi Buffer, 2 mM of 25 mM MgCl₂, 200 µM of 10 mM dNTPs mix, 1.0 U of Taq DNA Polymerase. The composition of master mix was with initial denaturation at 95°C for 5 min then denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s and strand extension at 72°C for 45 s for 34 cycles and a final extension at 72°C for 10 min. Similarly, the AS-PCR (on nested product of larva as template) was also run on the same conditions as that on the adult samples. The PCR amplicons

were checked for amplification by electrophoresis on a 1.5% agarose gel in TBE (1×) buffer at 120 V for 1h. The DNA migration and resolution pattern was examined by UV transillumination [Syngene Gel documentation system (UK)] technique and the picture was documented by photography. Frequencies of different genotype and alleles were calculated as per the method given by Pierce (2003).

RESULTS AND DISCUSSION

Primary and nested PCR: In the present study, a total of 50 adult parasites (25 each of male and female) and 138 larvae of *H. contortus* were genotyped for BZ resistance. The analyses of amplified product obtained after running primary and nested PCR on 1.0% Agarose gel in 1× TBE buffer showed bands at 939 bp and 820 bp, respectively. In case of larvae, the low concentrations of amplified primary product could not be demonstrated on agarose gel electrophoresis.

PCR-Restriction Fragment Length Polymorphism (PCR-RFLP): PCR-RFLP was performed by subjecting nested product to *RsaI* enzyme digestion thus generating three fragments of the size 462 bp, 211 bp and 147 bp.

Allele Specific PCR for detection of benzimidazole resistance: Analysis of AS-PCR products revealed a susceptible specific band of approximately 603 bp from susceptible larvae, whereas resistant larvae revealed a resistant specific band of approximately 222 bp. Besides this a non specific band of 774 bp was also observed in some cases. In heterozygous, all the three bands of approximately 774 bp, 603 bp and 222 bp were observed. Genotyped adult *H. contortus* were found to be homozygous resistant (rr) (Table 1) showing 100% prevalence of resistant allele (r). Tiwari *et al.* (2006) observed the AR in BZ in 162 adult male *H. contortus* of sheep collected from three different farms of Rajasthan and found the overall prevalence of BZ resistant allele (r) was higher (87%) as compared to 13% of BZ susceptible allele (S). The results indicated that there was very high frequency of homozygous resistant (rr) population and very low population of homozygous susceptible (SS). The high level of BZ resistance in adult parasites collected from abattoir indicated that the survival population is of resistant worms to the benzimidazoles anthelmintic predicted the judicious usage of the anthelmintic treatments. AS-PCR employed on the third stage larvae revealed different genotypic and allelic frequencies in different districts as given in Table 2. The order of percentage of homozygous resistant (rr) frequencies in 6 districts were Bathinda (76%), Mansa (59.09%),

Table 1. Genotyping of adult *Haemonchus contortus* of sheep using AS-PCR for benzimidazole resistance

Location of abattoir	No. of worms	Genotyping (%)			Gene frequency	
		Resistant (rr)	Susceptible (SS)	Heterozygous (rS)	Resistant (r)	Susceptible (S)
Ludhiana	25	25 (100)	—	—	1.0	—
	25	25 (100)	—	—	1.0	—

Table 2. Genotyping of *Haemonchus contortus* larvae using AS-PCR for benzimidazole resistance

Location	No. of larvae genotyped	Genotyping (%)			Gene Frequency	
		Resistant (rr)	Susceptible (SS)	Heterozygous (rS)	Resistant (r)	Susceptible (S)
Barnala	23	6 (26.08)	2 (8.69)	15 (65.21)	0.586	0.414
Bathinda	25	19 (76.00)	1 (4)	5 (20)	0.86	0.14
Mansa	22	13 (59.09)	2 (9.09)	7 (31.81)	0.75	0.25
Moga	24	10 (41.67)	—	14 (58.33)	0.708	0.292
Muktsar	20	9 (45.00)	—	11 (55)	0.725	0.275
Sangrur	24	12 (50.00)	—	12 (50)	0.75	0.25
Overall	138	69 (49.28)	5 (3.62)	64 (46.37)	0.72	0.28

Sangrur (50%), Muktsar (45%), Moga (41.67%), and in Barnala (26.08%). Amongst the 6 districts, only three districts, viz. Barnala, Bathinda and Mansa accounted for the presence of meagre homozygous susceptible (SS) population of 8.69%, 4.00% and 9.09%, respectively. The heterozygous (rS) population revealed variable frequencies in the order Barnala (65.21%) followed by Moga (58.33%), Muktsar (55%), Sangrur (50%), Mansa (31.81%) and Bathinda (4.00%). An overall, genotyping of the larval *H. contortus* revealed 49.28% (69), homozygous resistant (rr), 3.62% (5) homozygous susceptible (SS) and 46.37% (64) heterozygous (rS) among different districts of western zone of Punjab. Chandra *et al.* (2015) recorded 55–85% of *H. contortus* rr, 10–21% SS and 5–24% rS among different regions of Uttar Pradesh. The allele frequencies were 67–87.5% for resistant and 12.5–33% for susceptible. However, it was revealed that the rr population were observed maximum in organised farms, however, in backyard farms rr population was emerging which varied from 67% to 74% in eastern Uttar Pradesh.

Diagnosis of BZ resistance by molecular techniques has been carried out by some research workers in other states of the country (Tiwari *et al.* 2006; Garg and Yadav 2009; Chandra *et al.* 2015), mainly on larval populations of small ruminants reared on intensive management system and / or kept at organised farms. It can be inferred that the animals under the farm conditions are comparatively dewormed more frequently than the private flocks leading to high resistance in them. Also, the geo-climatic conditions and managemental practices followed in the organized farms have a direct bearing on the benzimidazole resistance in the animals (Dixit *et al.* 2017). The AS-PCR strategy used in the present study could detect only the mutation F200Y in β -tubulin gene but it cannot detect other mutations which may be responsible for development of benzimidazole resistance, viz. F167Y (Prichard 2001) and E198A (Ghisi *et al.* 2007).

In the present study, higher frequency of homozygous resistant (rr) and heterozygous genotypes (rS) suggests that the rate of development of resistance in this area is increasing at a faster rate. There is continuous and indiscriminate use of the broad spectrum anthelmintics (mainly benzimidazole group of drugs) leading to high frequency of heterozygous (46.42%) individuals and if the corrective measures, viz. strategic dosing, change of

anthelmintic, correct dose, use of perfect drenching devices, use of standard drugs, correct drug for target worms and effective management practices are not adopted than shortly the nematodes of this area will enter in resistant phase with high frequency of homozygous resistant (rr) individuals. Also, regular monitoring of the parasitic count (egg per gram of faeces) should be done and accordingly treatment regimes are to be followed. To eliminate chances of developing cross-resistance, periodical rotation of anti-parasitic drugs with different chemical formulations and mode of actions seems essential.

In conclusion, the present study on small population of the parasites signifies that high level of anthelmintic resistance exists in *H. contortus* in western zone of the state. To access the exact scenario of the resistance against anthelmintics in GIT parasites especially *H. contortus* there is a need for further elaborative investigation in rest agroclimatic zones of the Punjab state on huge worm population.

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