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ORIGINAL ARTICLE

Polymorphism of Isotype 1 β-tubulin Gene of *Haemonchus contortus* -A Report

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Abstract

Nucleotide sequence analysis of isotype 1 β-tubulin gene of *Haemonchus contortus* collected from Uttarkashi, Pantnagar and Nainital was carried out to detect single nucleotide polymorphisms (SNP) in the gene associated with anthelmintic resistance. Nucleotide and amino acid sequence analysis of the fragments of the β-tubulin revealed mutation Phenylalanine-200 to Tyrosine (F200Y) in the isolates from Uttarkashi and Pantnagar while Phenylalanine was detected at codon 200 in the isolates from Nainital. The β-tubulin mutation at Glutamate-198 to Alanine (E198A) and Phenylalanine-167 to Tyrosine (F167Y) was not detected in any of the isolates. This shows that isolates from Uttarkashi and Pantnagar were resistant containing mutation F200Y. The isolate from Nainital was susceptible with none of the SNP at codon 200, 198 or 167. For effective worm control, it is important to know the status of anthelmintic efficacy in a particular agro-climatic zone. This will not only delay spread and rise of benzimidazole resistance in Uttarakhand but also the life span of usage of these benzimidazoles could be extended.

Key words: *Haemonchus contortus*, Sheep, β-tubulin gene, Benzimidazole, Resistance.

1. Introduction

Among livestock, small ruminant industry, mainly sheep and goats, plays a significant role in terms of generating economic security, wealth and stability among many communities in developing and the developed world. In small ruminants, parasitism is one of the most important causes of production losses around the world (Molento, 2009). *Haemonchus contortus* is economically the most important gastro-intestinal nematode (GIN) in subtropical and tropical countries; while in temperate regions, the principal one is *Teladorsagia circumcincta* (Taylor *et al.*, 2007). These parasites have detrimental effect on animal health (Luscher *et al*., 2005) leading to clinical and sub-clinical diseases, that may result in huge economic losses due to reduced weight gains, decreased and poor quality of wool and reduced animal productivity in general (Melhorn, 2008).

The use of anthelmintics remains the primary control strategy where different drugs are being used against mature and immature stages of virtually all important nematodes (Kohler, 2001). Compulsory and often excessive use of these drugs (Hein and Harrison, 2005) along with poor management practices (Wolstenholme *et al*., 2004), improper dose rate, non target approach etc. has resulted in emergence of anthelmintic resistance.

In India, the first report of anthelmintic resistance was documented from Uttarakhand, against phenothiazene and thiabendazole in *H. contortus* at State Sheep and Wool Research Station, Pashulok, Rishikesh (Varshney and Singh, 1976). Since then, benzimidazole resistance has been reported time and again in sheep populations of Uttarakhand (Kumar and Yadav, 1994; Garg and Yadav, 2009; Pandey, 2011; Baghel *et al.,* 2012; Rialch *et al.,* 2013). Reports regarding genetic variability of isotype 1 β-tubulin gene of *H. contortus* of small ruminants are scarce (Baghel *et al.,* 2012). Increase in the emergence of benzimidazole resistance demands extensive study of the related factors even at molecular levels, so that its further spread can be controlled early.

Keeping the above background information in mind and understanding the potential threat posed by widespread emergence of resistance to sustainable small ruminant economy in India and Uttarakhand in particular, the present investigation has been planned with the objective to study polymorphism of isotype 1 β-tubulin gene in *H. contortus* of sheep of different agro-climatic zones of Uttarakhand.

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2. Materials and Methods

2.1 Collection of Parasites

Adult male *H. contortus* were isolated from abomasum of sheep slaughtered at Uttarkashi and Nainital. The parasites were also collected from a sheep that had died in Department of Veterinary Parasitology, College of Veterinary and Animal Sciences, Pantnagar.

2.2 Genomic DNA Isolation

Genomic DNA was isolated from single adult male worm separately on individual basis as per the method described by Anonymous (1999) with modifications. The worms were thoroughly washed in distilled water. Individual worm was collected and suspended in 500µl of worm lysis solution (100 mM Tris-Cl, 100 mM NaCl, 50 mM EDTA, 1% SDS, 1% β-mercaptoethanol, 100µg/ml Proteinase K and 20µg/ml RNaseA) in separate tubes and frozen at -60ºC for 1 hour. The tubes were then thawed and incubated at 65ºC for 1 hour with occasional agitation.

After incubation, 500µl mixture of phenol: chloroform: isoamyl-alcohol (25:24:1) (Amresco, USA) was added to each tube. The contents of the tubes were mixed gently by inverting the tubes till a homogenous white solution was visible and then centrifuged at 7826×g for 10 minutes at 4ºC. 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 were mixed and centrifuged again at 7826×g for 10 minutes at 4ºC.

The aqueous phase thus formed in each tube was again collected in separate tubes and 500µl of chilled absolute ethanol (Commercial Alcohols Inc., Germany) and 100µl of 5M sodium acetate (Biogene, USA) were added. The contents of each tube were mixed and then all tubes were kept at -20ºC for 1 hour followed by centrifugation at 11269×g for 15 minutes. The resulting supernatant in each tube was discarded and the DNA pellet was mixed with 70% ethanol. It was again centrifuged at 7826×g for 3-4 minutes at 4ºC and the supernatant was discarded.

The washed DNA pellet was air dried in open tubes at room temperature for 30 minutes to remove traces of ethanol and then suspended in 30µl of TE buffer (10 mM Tris-Cl and 1mM EDTA, pH 8.0). The DNA samples were kept at -20ºC till further use. To determine the purity and concentration of the isolated DNA, UV/VIS spectrophotometry was used. The purity was estimated by determining the ratio of A_{260} and A_{280} (1.8 for a pure DNA sample).

2.3 Agarose Gel Electrophoresis

A submarine horizontal electrophoresis unit (Atto, Japan) was used to further determine the resolution and purity of isolated DNA samples and the technique used was agarose gel electrophoresis. 0.8% agarose gel was prepared by boiling molecular grade agarose (Life Technologies, India) in a 1X TBE buffer to dissolve it completely. Solution was allowed to cool to about 50ºC and ethidium bromide (Amresco, USA) was added to agarose solution to obtain final concentration of 0.5µg/ml.

Each DNA sample (5 µl) was mixed with 2 µl of 6X gel loading dye and loaded into the wells along with 10Kbp DNA ladder (Invitrogen, USA). Electrophoresis was performed at 5 volts/cm for about 90 minutes and progress of mobility was periodically monitored by migration of the dye. The migration and resolution of DNA samples were examined by UV transillumination technique and the photograph and size of DNA were documented using a gel documentation system (Lambda 35, Perkin Elmer, Germany).

2.4 Amplification of β-tubulin Gene

The isotype 1 β-tubulin gene of each DNA sample was amplified by PCR using the oligonucleotide primers (Genei, Bangalore):

P_1 : 5' GGC AAA TAT GTC CCA CGT GC 3'
P_2 : 5' GAT CAG CAT TCA GCT GTC CA 3'

PCR was carried out in thin wall PCR tubes in 50µl reaction volume. Genomic DNA samples from adult *H. contortus* were used as templates for amplification of β-tubulin gene in separate tubes. The PCR mixture consisted of 5µl of DNA template (about 50 ng DNA), 5µl of 10X PCR buffer (minus Mg), 0.30mM of each dNTP (Invitrogen, USA), 20 pmol of each primer (P_1 and P_2), 3mM $MgCl_2$ and 3.0U of Taq DNA polymerase (Invitrogen, Brazil). The volume of the reaction mixture was made up to 50µl with autoclaved milli-Q water. Polymerase chain reaction was performed in an automated programmed manner using EP-Gradient S thermocycler (Eppendorf, Germany) with the following conditions: Initial denaturation at 94ºC for 5 minutes was followed by 30 cycles each of denaturation at 94ºC for 1 minute, annealing at 56.5ºC for 1 minute and extension at 72ºC for 1 minute. This was followed by final extension for 10 minutes at 72ºC. The PCR tubes were properly labelled and stored at -20ºC till further use. The β-tubulin PCR amplions were analysed by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide.

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2.5 Nucleotide Sequencing and Polymorphism of β-tubulin Isotype 1 Gene of *H. contortus*

Three PCR products (1 each from Uttarkashi, Pantnagar and Nainital) were sent to SciGenome Labs Pvt Ltd., Cochin; Kerela for nucleotide sequencing (Double pass analysis using forward and reverse primer). Correct sequence for all the three PCR products were determined individually from the electropherogram and sequence data obtained using both forward and reverse primers. Amino acid sequence was deduced from the nucleotide sequences using the BioEdit software.

For β-tubulin isotype 1 and SNP verification at position 200, 198 and 167, the translated nucleotide sequences were compared and analysed using the known resistant and susceptible *H. contortus* sequences available in the GenBank and the resistance and susceptibility status was thus established.

3. Results and Discussion

The genomic DNA from adult *H. contortus* was extracted using phenol: chloroform: isoamyl alcohol and the average A_{260}:A_{280} ratio of extracted DNA from individual worms was 1.813 indicating high purity of DNA without RNA. The concentration of DNA ranged from 1.478-1.537 µg/ml. The quantity of DNA was further evaluated by agarose gel electrophoresis using 0.8% agarose gel (Fig 1).

Amplification of β-tubulin isotype 1 gene of each DNA sample yielded partial β-tubulin gene PCR product of approximately 840 bp (Fig 2) in all the cases. The nucleic acid sequences of three PCR products of amplified partial β-tubulin isotype 1 gene of *H. contortus* collected from three different regions i.e. Uttarkashi, Pantnagar and Nainital were obtained.

3.1 Detecting Benzimidazole Resistance Status by Studying Single Nucleotide Polymorphism of β-tubulin Isotype 1 Gene of *H. contortus*

For β-tubulin isotype 1 and SNP verification at position 200, 198 and 167, the deduced nucleotide sequences were compared and analysed using the known resistant and susceptible *H. contortus* sequences available in the GenBank and the resistance and susceptibility status was thus established. The Blast (Altschul *et al.,* 1990) results verified that the amplified fragments were 100% *H. contortus* partial β-tubulin isotype 1 gene. Nucleotide and amino acid sequence analysis of the fragments verified the presence of both wild type TTC and the TAC SNP and the Phe to Tyr amino acid change.

The β-tubulin mutation Phenylalanine-200 to Tyrosine (F200Y) was detected in the isolates from Uttarkashi and Pantnagar while Phenylalanine was detected at codon 200 in the isolates from Nainital (Fig 3). The β-tubulin mutations at Glutamate-198 to Alanine (E198A) or Phenylalanine-167 to Tyrosine (F167Y) were not detected in any of the isolates from these three regions and were containing Glutamate and Phenylalanine, respectively (Fig 4). This shows that isolates from Uttarkashi and Pantnagar were resistant containing mutation F200Y. While the isolate from Nainital was detected susceptible with none of the SNP at codon 200, 198 or 167.

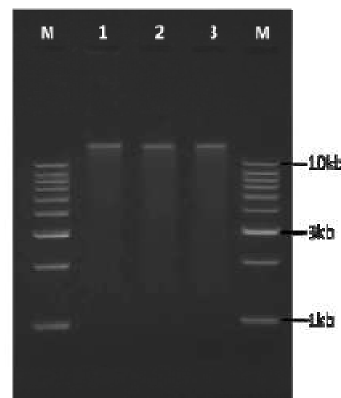

Fig 1: *Haemonchus contortus* (Adult) genomic DNA

Lane M : 1 Kb DNA ladder
Lane 1 : Isolate from Uttarkashi
Lane 2 : Isolate from Pantnagar
Lane 3 : Isolate from Nainital

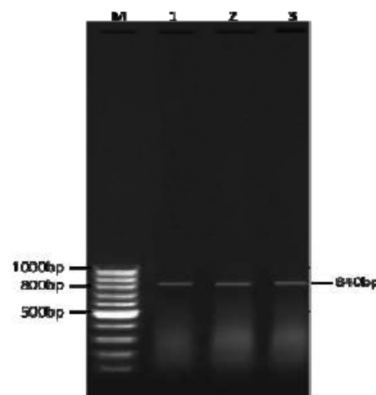

Fig 2: Amplified Beta tubulin gene from adult *Haemonchus contortus*

Lane M : 100 bp DNA ladder
Lane 1 : Isolate from Uttarkashi
Lane 2 : Isolate from Pantnagar
Lane 3 : Isolate from Nainital

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						198		200					
DQ873734.1	V	E	N	T	D	E	T	F	C	I	D	N	E
AF157216.1	V	E	N	T	D	E	T	Y	C	I	D	N	E
ACS29569.1	V	E	N	T	D	A	T	F	C	I	D	N	E
Uttarkashi	V	E	N	T	D	E	T	Y	C	I	D	N	E
Pantnagar	V	E	N	T	D	E	T	Y	C	I	D	N	E
Nainital	V	K	N	T	D	E	T	F	C	I	D	N	E

Fig 3: Alignment from amino acid 193-205 of β-tubulin isotype 1 gene from BZ- susceptible *Haemonchus contortus* (GenBank accession number DQ873734.1), BZ-resistant *H. contortus* with F200Y (AF157216) or with E198A (ACS29569.1) and from adult *H. contortus* isolates from Uttarkashi, Pantnagar and Nainital

							167			
DQ873734.1	D	R	I	M	A	S	F	S	V	V
ACY00537.1	D	R	I	M	A	S	Y	S	V	V
Uttarkashi	D	R	I	M	A	S	F	S	V	V
Pantnagar	D	R	I	M	A	S	F	S	V	V
Nainital	D	R	I	M	A	S	F	S	V	V

Fig 4: Alignment from amino acid 161-170 of β-tubulin isotype 1 gene from BZ- susceptible *Haemonchus contortus* (GenBank accession number DQ873734.1), BZ-resistant *H. contortus* with F167Y (ACY00537.1) and from adult *H. contortus* isolates from Uttarkashi, Pantnagar and Nainital

Earlier also BZ resistance has been reported from Pantnagar of Tarai region as compared to Mukteswar and Kedarkattha of Hill region of Uttarakhand (Garg and Yadav, 2009). Detection of SNP F200Y in Pantnagar might be due to genetic selection of the resistant worms present in population due to high selection pressure resulting because of frequent and indiscriminate use of anthelmintics (Garg and Yadav, 2009).

SNP F200Y was not detected in worms in Nainital. This is a high altitude area 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 (Garg and Yadav, 2009). In Uttarkashi, yet another high altitude region in hills, SNP F200Y of partial beta tubulin gene isotype-1 was observed in *Haemonchus*. This might be due to the migration of animals from hills to plain areas from where the animals might have picked up the resistant worm population. Uttarakhand livestock owners keep migratory flocks of sheep which move downward into Tarai regions during colder months of the year. Thus parasite populations tend to be mobile with movement of flocks further adding to spilling of resistant parasites to regions hitherto not exhibiting emergence of benzimidazoles resistance.

Molecular techniques are of importance in diagnosis of BZ resistance. While FECRT and EHA give an estimate of the anthelmintic resistance of the whole worm community (Humbert *et al.,* 2001), molecular techniques can detect even emergence of resistance i.e. when less than 25% worms are resistant in a population (Martin *et al.,* 1989).

The most common molecular mechanism conferring BZ resistance in *H. contortus* in small ruminants involves a Phenylalanine (TTC) to Tyrosine (TAC) mutation at residue 200 of isotype 1 and 2 β-tubulin genes (Kwa *et al.,* 1994; Elard *et al.,* 1996, 1999; Tiwari *et al.,* 2006). Other mutations associated with BZ resistance in β-tubulin is Phenylalanine-167 to Tyrosine (F200Y) and Glutamate-198 to Alanine (E198A) (Ghisi *et al.,* 2007). Garg (2006) also observed F200Y at residue 193 of isotype 1 β-tubulin gene in resistant *H. contortus* isolates from Pantnagar and Mukteshwar. In addition to above, there had been reports of difference in amino acid residues at 76 and 368 in susceptible and resistant strains of parasites (Kwa *et al.,* 1995) and recently, difference at 190 amino acid has also been reported (Miranda-Miranda *et al.,* 2008). Along with the above mentioned

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mechanisms, there are also certain non-specific mechanisms like, change in drug transport (Smith and Prichard, 2002) and alteration in drug metabolism (Kawalek *et al.,* 1984).

The exact matches with peptide sequences VENTDETFCIDNE (residues 193-205 of β-tubulin isotype 1 gene) and DRIMASFSVV (residues 161-170 of β-tubulin isotype 1 gene) were searched in translated Blast (tblastn; Altschul *et al.,* 1990) against GenBank's nucleotide sequences to compare the sequences obtained in the isolates in the study. Only one point mutation in β-tubulin could be correlated to detect BZ resistance (F200Y).

Although F200Y SNP in β-tubulin gene is the most commonly associated with BZ resistance in *H. contortus*, there are isolates that may possess two BZ resistance SNPs (E198A and F200Y) (Kotze *et al.,* 2012). Of these, E198A SNP confers higher levels of BZ resistance than F200Y SNP (Kotze *et al.,* 2012). The isotype 1 β-tubulin gene of *H. contortus* is highly conserved (Elard *et al.,* 1996) but isolates from different geo-climatic origins show polymorphism depending on mutation rates, population size and migration rates of parasite populations (Prichard, 2001). The high population size of *H. contortus,* high fecundity and ability to survive in different range of environmental conditions are responsible for extreme genetic diversity in the parasite.

For effective worm control, it is important to know the status of anthelmintic efficacy in a particular agro-climatic zone. The animal owners should be made aware of the proper dosage and appropriate time of administration of benzimidazoles to their animals. This will not only delay spread and rise of benzimidazole resistance in Uttarakhand but also the life span of usage of these benzimidazoles could be extended.

4. Conclusion

Genetic variability exists in different populations of *H. contortus* collected from Uttarakhand. For effective worm control, regular monitoring for anthelmintic resistance is important to know the status of anthelmintic efficacy in a particular agro-climatic zone. This will enable timely management of benzimidazole resistance in gastrointestinal nematodes.

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