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Genetic Variability in β-tubulin-1 in Benzimidazole Resistant *Haemonchus contortus* from Sheep in North-East Punjab, Pakistan

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Abstract.- Benzimidazole is a synthetic anthelmintic against which nematode resistance especially in *Haemonchus contortus*, is emerging at a alarming speed. The mechanism of benzimidazole resistance appears to involve mutations in the gene encoding β-tubulin isotype 1 (β-tubulin-1). The present study was carried out to find out the variation existing in β-tubulin-1 which is directly involved with drug binding capacity involving microtubules polymerization. DNA of adult nematode *H. contortus* was extracted, amplified and sequenced. Out of 50 worms investigated, 37 showed benzimidazole susceptible gene while 13 were resistant indicating single nucleotide mutation at amino acid 200 TTC/TAC. In addition, 12 worms showed several regions of consistent difference indicating single nucleotide polymorphism (SNPs) at various positions in coding region. It has been concluded that resistant alleles conferring anthelmintic resistance is prevalent in the local population of *H. contortus* of north-east Punjab, Pakistan.

Keywords: Genetic variability**,** Benzimidazole resistnace, β-tubulin-1 gene, nematode, *Haemonchus contortus*.

INTRODUCTION

Infection caused by gastrointestinal nematodes (GIN) is one of the major constraint in small ruminants production system of Pakistan. Each year, heavy production losses have been experienced due to mortality and morbidity in young animals in the Pothwar region of Pakistan. These problems have become severe due to existence of inadequate animal husbandry practices. *Haemonchus contortus* is a blood sucking neamatode causes anaemia and diarrhoea leading to death if not treated. This nematode is well-thrived in climatic environments ranging from tropical to cold mountaineous areas (Eckert and Hertzberg, 1994; Dorny *et al.*, 1996; Kuchai *et al*., 2012). In Pakistan the prevalence rate of *H. contortus* in sheep was found to be 25.1 to 92% in Pakistan (Tasawar *et al*., 2010).

One of the current controlling strategies is the application of chemotherapy in the form of anthelmintics. Benzimidazoles (BZ) is the most commonly used anthelmintic drug. It binds to β-tubulin and thus preventing polymerization of tubulin dimers to microtubules (Lacey, 1988). Microtubules constitute the infrastructure for direct intracellular transport through formation of a dynamic cytoskeleton (Caviston and Holzbaur, 2006). Three phenomena account for BZ resistance in a population; gene flow from one population to the other, previously existing resistant alleles and spontaneous mutations (Rymond *et al*., 1991; Medeiros, 1997; O'Brien, 1997). *H. contortus* exhibits wide genetic diversity as mutation rate in *H. contortus* is ten times greater as compared to vertebrates (Blouin *et al*., 1995). Genetic evidences indicate that certain residues of β-tubulin-1 are critical for BZ action although they are not part of the drug binding site. Two classes of β-tubulin have been identified and isotype 1, *i.e.* β-tubulin-1 is directly related to resistance. Genetic resistance to BZ in *H. contortus* involves mutations in β-tubulin genes at specific amino acid sites. The change in single nucleotide from tyrosine to alanine selects the

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individual for resistance. This mutation is also responsible for BZ resistance in other nematode species, including *Caenorhabditis elegans*, *Cylicocyclus nassatus* and *Cyathostomum coronatum* (Kwa *et al*., 1993; Kwa *et al*., 1994; Kwa *et al*., 1995; Pape *et al.*, 1999; Samson-Himmelstjerna *et al.*, 2001). The specific toxicity of BZ seems to be due to their affinity for helminth β-tubulins (Lacey, 1988).

Although there have been reports of indiscriminate use of BZ without following proper awareness and guidance lay down by livestock department, yet there is no previous knowledge exist on the molecular aspects of emerging anthelmintic resistance in *H. contortus*. This study was aimed to investigate genetic variation existing in β-tubulin-1 of the *H. contortus* population of sheep in north-east Punjab, Pakistan.

MATERIALS AND METHODS

Study area

The study area is climatically charaterized as sub-tropical zone. It is situated between latitude 30 and 34◦N and longitude 70 and 74◦E. The climate of the area is semi-arid, influenced generally by summer rainy monsoon (July–September) and partly from winter precipitation and well suitable for thriving gastrointestinal nematodes of sheep.

Parasite collection

Adult *H. contortus* were isolated directly from abomasa of sheep slaughtered at abattoir located in north-east Punjab, Pakistan. Sheep in the area are generally dewormed by albendazole. Fifty adult nematode worms were collected and identified morphologically according to Mohiuddin *et al*. (1984). They were kept in separate tubes containing 70% ethanol for later analysis.

DNA extraction and polymerase reaction

DNA was extracted from individual worms using QiAamp DNA Mini Kit (Qiagen, Valencia, CA) according to manufacturer instructions. The PCR reaction was carried out with total volume of 20 µl containing genomic DNA. Taq polymerase (2.4 unit), dNTPs (1.8 µl), $MgCl_2$ (1.5 mM) and 10x reaction buffer was used in automated thermocycler for 5 min at 95°C, followed by 35 cycles each of incubation at 94°C for 45s, annealing at 58°C for 45s and extension at 72°C for 45s. The amplification was completed with the additional extension step for 10 minutes. The PCR product was analyzed using 0.5% agarose gel in 0.5 TBE buffer and visualised using ethidium bromide and UV-transilluminator.

Purification and sequencing

The samples were purified using Qiaquick column (Qiagen) resulting in purified PCR product. The second gel was run to assess the quantity of DNA after the purification process before proceeding to sequencing reaction where the nucleotide sequences were labelled. The labelled product was precipitated and re-suspended in formamide ready for sequencing using 3130xl Genetic analyzer (Applied Biosystem). Chromatograms obtained can be interpreted for different alleles present in individual parasites. The polymorphic sites were identified by using the standard set of ambiguity nucleotides through CLC Genomics work bench (CLC bio) and BLAST searches at NCBI/primers used to generate the β-tubulin gene sequence from *H. contortus* with accession No. X80046 version X80046.1 G1: 897752, were as follow

β-tubulin	forward	5’gttctccgttgttccatcacc3’
	reverse	5’cgtgacaccagacattgtgacag3’

RESULTS

The isolated genomic DNA of *H. contortus* was used to amplify β-tubulin-1 gene. Out of 50 worms, 10 showed substantial amount of single nucleotide polymorphism (SNPs) when compared with the sequences in Gene Bank. Six polymorphic regions including some synonymous changes were observed. Thirteen sequences showed resistant alleles, while 37 were susceptible.

In susceptible nematodes, there was TTC in the coding region of the gene, while it was replaced with TAC for resistant individuals (Fig. 1) which resulted in

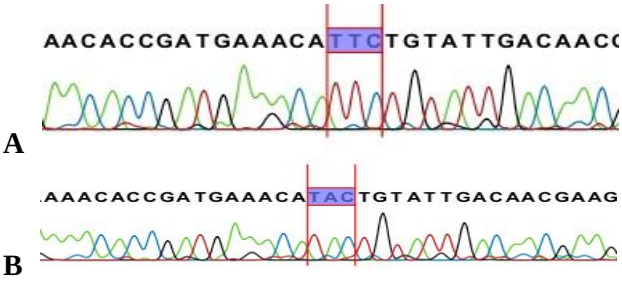

Fig. 1. Chromatogram of PCR product in the region of codon 200. The shaded portion indicates the susceptible allele in A, and resistant allele in B.

Table I. SNPs location at β-tubulin.

SNP position	Sequence change	Amino acid change	Amino acid position	SNP frequency
554	GTA > GTG	V = V	200	1
563	RGT > TGT	Y/C = C	188	2
579	GGG > GGA	G = G	193	3
594	GTG > GTA	V = V	198	5
675	TAT > TGT	Y = C	544	1
782	ACA > ATA	T = I	261	2
873	GGA > TGA	G > ●	137	1

change of amino acid from phenylalanine to tyrosine (F200Y). The β-tubulin replacement from phenyl alanine to tyrosine was observed in resistant sequences and all worms were homozygous for resistance/susceptible allele. Inter-specific variations observed are shown in Table I. Five parasites showed SNP at position 594 (position according to reference gene) which is the highest degree of substitution in this experiment replacing G with A coding for peptide at codon 198 in Genome Bank, while there was no change in amino acid (V = V) and account for 10% of total polymorphic changes. Such a substitution mutation can be impotant because of its effect on mRNA stability, its transport or its translation due to codon usage bias. One worm showed important substitution at position 873 (GGA/TGA) where G is replaced with T and amino acid glycine was changed to stop codon. Two sequences showed polymorphism at nucleotide 563 where R is representing either A or G (both A and G peaks in chromatograms were observed) and nucleotide position 579 where G replaced A, but there was no change in protein translation. Two sequences showed the change in codon 782 changing tyrosine to isoleucine (T261I). Forty (40) parasite sequences exactly matched (100%) that of isotype 1 of *H. contortus* over the region amplified gene bank GQ910916, while others showed varying degree of resemblance to the sequences available in data base.

DISCUSSION

BZ resistance is routinely detected by various *in vivo* and *in vitro* assays including fecal egg count reduction test, egg hatch and larval development assays (Le Jambre, 1976; Coles *et al.*, 1992). However, these assays are time consuming, expensive and less sensitive as compared to recently developed molecular techniques which are highly sensitive and economical for diagnosis of anthelmintic resistance. Using molecular techniques, BZ resistance has been mainly linked with the mutation in β-tubulin isotype 1 gene which substitutes phenylalanine (Phe) with tyrosine (Tyr) at the 200 codon position (Tiwari *et al.*, 2007). Previously no information is generated on the molecular aspect of BZ resistance for *H. contortus.*It is to be pointed out that understanding the molecular mechanisms involved in drug resistance may suggest more effective strategies to control haemonchosis in small ruminants.

There are several molecular markers used to detect anthelmintic resistance yet SNPs as diagnostic tool yield more reliable results (Corley and Jarmon, 2012). In present study BZ resistance have suggested the occurrence of pre-existing alleles in *H. contortus* population in addition to new allele generated as result of spontaneous mutation. The mutated allele TAC detected in the present study has already been reported (Corley and Jarmon, 2012). Sequencing of β-tubulin-1 in variety of fungal genera and closely related filarial parasites has demonstrated the same single amino acid substitution at position 200 in the resistant strains (Guenette *et al.*, 1991). Thus it can be concluded that region of BZ resistance is same in phylogenetically-distant organisms *viz.*, fungi to nematodes. There is also genetic evidence that

resistance is outcome of single, sex linked, gene in *T. columbriformis* and *O. dentatum*, but in *H. contortus* multiple genes could be involved (Sangster *et al.*, 2002). Our results correspond to an earlier report (Tiwari *et al.*, 2007).

We have also detected a mutation at amino acid position 198 as mentioned previously (Shokrani *et al*., 2012). It lies close to the resistance/susceptible allele thus can play an important role in shaping some conformational changes in protein structure. Some allelic positions showing differences in nucleotide sequence in the current study do not correspond to any previously identified nucleotides involved in resistance. However, it is yet to be determined whether amino acid change at relatively close residues such as the one observed at position 198, would have an effect on binding site of drug that may enhance anthelmintic resistance. Interestingly, the mutation 167 reported in previous studies (Shokrani *et al*., 2012) has not been observed in any of the sequence in the present study which indicates that this change is not common in the population studied. Some studies indicate the presence of this allele and its association with resistance but other studies donot support this data. Therefore it may warrant further genetic analysis in future investigations.

The present results demonstrate that SNP detection can be useful tool in finding out whether sheep are harboring benzimidazole susceptible or resistant *H. contortus* strains. Moreover, molecular diagnosis technique is a good approach to advance typical procedures of screening and checking the progress of anthelmintic resistance in small ruminants. Molecular detection can also benefit the livestock sector especially pharmaceutical enterprises to target *H. contortus* at the molecular level and consequently enhance anthelmintic remedies to cure nematodes especially *H. contortus.*

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