

Original Research Article

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Molecular Characterization of β -Tubulin Isotype-1 Gene of *Bunostomum trigonocephalum*

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ABSTRACT

The mechanism of benzimidazoles resistance is linked to single nucleotide polymorphisms (SNPs) on beta-tubulin isotype-1 gene. The three known SNPs responsible for BZ resistance are F200Y, F167Y and E198A on the beta-tubulin isotype-1. The present study was aimed to characterize beta-tubulin isotype-1 gene of *Bunostomum trigonocephalum*, for identifying variations on possible mutation sites. The adult parasites were collected from Mukteswar, Uttarakhand. The parasites were thoroughly examined morphologically and male parasites were subjected for RNA isolation. Complementary DNA (cDNA) was synthesised from total RNA using OdT. The PCR was performed using cDNA and self designed degenerative primers. The purified PCR amplicons were cloned into pGEMT easy vector and custom sequenced. The obtained sequences were analysed using DNA STAR, MEGA7.0 and Gene tool software. The deduced amino acid sequence showed 99% homology with published *B. trigonocephalum* as well as closely related nematodes *Ancylostomum caninum* and *Strongyles* of equines. It is also showed 98-98.7% identity with *Trichostrongylus* species and 91.8-93.6% homology with other helminths like *P.equorum*, *A. galli* and *F.hepatica*. The information obtained from current study enlighten to investigate further related to benzimidazole resistance in *B. trigonocephalum* and formulate effective control strategy as this parasite is one of the most pathogenic strongyles of small ruminants.

Keywords

Benzimidazole resistance, Beta tubulin, *Bunostomum trigonocephalum*, Small ruminants

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Introduction

Bunostomum trigonocephalum (Order: Strongylida, Family: Ancylostomatidae) is commonly known as hookworm of small

ruminants and the infection, bunostomiasis is characterised by anaemia due to blood sucking of worm and dermatitis by larval penetration, particularly lower part of infected host (Soulsby, 1982). Few hundreds of worm can

kill an animal (Soulsby, 1982) and Bunostomiasis is reported to be affecting all age groups, mainly of growing young ones (5-8 months aged). The infection is more prevalent in warm and humid regions (Tariq *et al.*, 2008, 2010), and is also reported as major cause of economic losses in the livestock industry in temperate areas (Stancampiano, 2007). The prevalence of *Bunostomum* spp. is restricted in few pockets of India, mainly from central (Singh *et al.*, 2016; Rajpoot *et al.*, 2017), north east (Yadav and Tondon, 1989; Bandyopadhyay *et al.*, 2010) and Kashmir valley (Tariq *et al.*, 2008, 2010). The prevalence of *B. trigonocephalum* is very high, especially in Kashmir valley, where prevalence in sheep and goat were 37.7% and 30.1%, respectively (Tariq *et al.*, 2008, 2010). Control of gastrointestinal nematodiasis including bunostomiasis has achieved by using broad spectrum chemotherapeutic agents like benzimidazoles (BZs), imidazothiazoles, tetrahydropyrimidines and macrocyclic lactones. The excessive and frequent use of anthelmintics has resulted in substantial and widespread emergence of anthelmintic resistance (AR), particularly against BZ in nematode populations (Kaplan *et al.*, 2004; Garg and Yadav, 2009; Chandra *et al.*, 2014, 2015; Dixit *et al.*, 2017). Maximum reports of BZ resistance are restricted in three main gastrointestinal nematodes *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* (Kwa *et al.*, 1993, 1994, 1995; Silvestre and Humbert, 2000; Ghisi *et al.*, 2007; Rufener *et al.*, 2009; Garg and Yadav, 2009; Chandra *et al.*, 2014, 2015; Dixit *et al.*, 2017). BZ resistance is primarily linked to a point mutation at amino acid 200 (Phe to Tyr) (Kwa *et al.*, 1993, 1994, 1995), 167 (Phe to Tyr) (Ghisi *et al.*, 2007) and 198 (Glu to Ala) (Rufener *et al.*, 2009, Chaudhary *et al.*, 2015) of β -tubulin isotype-1 gene. However, works on *Bunostomum* genus, particularly on the *B. trigonocephalum* is meager. Therefore, it is necessary to

characterize β -tubulin gene of *B. trigonocephalum* for analyzing and predicting mutation pattern with respect to BZ resistance. With this aim, the present study was planned to characterize beta-tubulin isotype 1 gene of *B. trigonocephalum* of Mukteswar.

Materials and Methods

Study area and collection of parasites

Adult *Bunostomum trigonocephalum* isolate were collected from gastrointestinal tract of goats slaughtered at local abattoir at Mukteswar (29°28'N and 79°39'E, 7500 feet above mean sea level), Uttarakhand. Parasites were washed thoroughly in PBS (pH 7.4) and identified as per the morphological keys (Johnson, 1965; Soulsby, 1982). The adult male worms were used for extraction of total RNA.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from adult male *B. trigonocephalum* using RNeasy minikit (Qiagen, Germany) as per manufacturer's instructions. The complementary DNA (cDNA) was synthesized from the total RNA of adult male *B. trigonocephalum* using oligo dT primer and by using RevertAid reverse transcriptase enzyme (Thermo scientific, USA).

Polymerase chain reaction for amplification of β -tubulin isotype-1 gene

PCR was standardized to amplify the β -tubulin isotype-1 gene of *B. trigonocephalum* from cDNA. The open reading frame of truncated β -tubulin gene was amplified using the self-designed degenerative primers (forward primer 5'GCC GGW CAR TGC GGH AAC CAG 3' and reverse primer 5'GTG AAY TCC ATT TCG TCC ATA C 3') and were

designed to amplify all the expected mutations for BZ resistance present in the gene such as 167th, 198th and 200th position. The PCR mixture consisted of cDNA as template 1.0µl, TerraTM PCR Direct Red Dye Premix 12.5 µl, 10 pmoles of each primer and adding nuclease water to make final volume 25 µl. The reaction was standardized with annealing temperature at 60°C. The amplicons were confirmed in 1.2% agarose gel electrophoresis.

Molecular cloning, sequencing and characterization of β -tubulin gene of *B.trigonocephalum*

The amplicons were gel purified using Qiaquick Gel extraction kit (Qiagen, Germany) and ligated with 50ng of pGEM®-T easy TA cloning vector (Promega) in 1:3 ratios (Vector: Purified amplicons). The recombinant plasmid was transformed in to *E.coli* Top10 competent cells by heat-shock method at 42°C for 90 sec. The transformed culture was plated over the freshly prepared LB Amp+ X gal+ IPTG+ plates and incubated overnight at 37°C. The positive colonies were selected using blue-white screening method (α -complementation) and further confirmed by colony PCR and release of desired products from vector using *EcoRI* enzyme by restriction enzyme digestion. Subsequently, the positive clone was inoculated in LB stab culture and custom sequenced.

Genetic characterization

Stab cultures of positive clones harbouring the desired β -tubulin gene was sent for custom DNA sequencing to Department of Biochemistry, Delhi University, South campus. The sequence information received was analyzed by using ClustalW pair distance method (DNA Star) and phylogenetic tree was constructed using maximum likelihood method (MEGA version 7.0) with published beta tubulin isotype 1 gene of

B.trigonocephalum and other related *Strongylus* species. The β -tubulin gene sequences of other *Strongylus* and other helminthes were retrieved from NCBI database and used for comparative analysis purpose.

Results and Discussion

The anterior end of *B.trigonocephalum* is bent in a dorsal direction; therefore the parasite is looks like hooks. The buccal capsule is triangular funnel shape opens antero-dorsally; it has a large dorsal tooth and two short ventral teeth. There are two sub-ventral cutting plates and small pair of dorsal plates near moth opening. Large dorsalcone is characteristics of this species, which projects in to the $\frac{2}{3}$ of buccal cavity (Fig.1). The bursa of male *B.trigonocephalum* is well developed with small asymmetrical dorsal lobe, which is not well demarcated from lateral lobes. The spicules are spirally twisted and united posteriorly. The spicules are 0.6-0.64 mm long, slender and alate. The gubernaculum is absent (Fig.2). The morphological features of *B.trigonocephalum* are documented elsewhere (Johnson, 1965; Soulsby, 1982; Suresh Singh, 2003)

Amplification of β -tubulin gene sequence of *B.trigonocephalum*

The PCR was amplified approximately 1178 bp size fragment of β -tubulin isotype-1 gene in agarose gel electrophoresis (Fig.3). The PCR product was purified and the concentration of purified β -tubulin gene was 32ng/µl.

The ligated amplicon with pGEM®-T easy TA cloning vector was successfully transformed as evidenced by appearance of desired white colonies in the LB Amp+ X gal+ IPTG+ plates and by colony PCR (Fig.4). The presence of insert was further confirmed by restriction enzyme analysis (Fig.5).

Sequencing and genetic characterization of β -tubulin isotype-1 gene of *B. trigonocephalum*

The positive clones harboring β -tubulin gene of *B. trigonocephalum* were custom sequenced and analysis result revealed that the amplicon size is 1178 bp. The deduced amino acid of sequenced region of the gene consisted of all the possible and reported mutation sites for BZ resistance i.e. F167Y, E198A and F200Y. Mutation at amino acid 200 of the beta tubulin isotype-1 (Phe to Tyr) is mostly responsible for BZ resistance in *H. contortus* (Kwa *et al.*, 1994; Rufener *et al.*, 2009). However, mutations at 167 (Phe to Tyr) and 198 (Glu to Ala) are also reported to be associated with resistance in some isolates of *H. contortus* (Prichard, 2001; Ghisi *et al.*, 2007; Rufener *et al.*, 2009).

The characterization of beta tubulin isotype-1 gene of *B. trigonocephalum* revealed that the organism is susceptible to benzimidazole resistance as amino acid on 167 and 200th position is phenylalanine, and 198th position is glutamic acid. The sequenced genes were aligned and analysed with available β - tubulin gene sequence of *B. trigonocephalum* and other *Strongylus* sequences (Fig.6). The beta-tubulin isotype-1 gene of *B. trigonocephalum* has >99% with published sequence of same organism and much closed related parasites *A. caninum* and *Cyathostomes*.

The identity was 98-98.7% with *Trichostrongylus* species such as *H. contortus*, *T. colubriformis*, *Cooperia pectinata* and *C. oncophora*. Further, 91.8-93.6% homology with other species of helminths like *P. equorum*, *A. galli* and *F. hepatica*. Since the β -tubulin is one of the framework proteins of the cell function, many of the amino acid will be conserved. Therefore, single mutation creates considerable functional consequences.

Phylogenetic tree was constructed with Maximum Likelihood (ML) method using Tamura-Nei model with 500 bootstrap replications to confirm the authenticity of the taxa analysed for each node. Input file was obtained by applying the BioNJ method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach.

A discrete Gamma distribution was used to model evolutionary rate differences and number of substitutions per site (Fig.7). For understanding the real situation of BZ resistance at field level, characterization of beta tubulin gene is pre-requisite. Characterization studies on target genes enable to identify their polymorphism, if any, which may provide great platform in formulating effective control strategies. The phylogenetic analysis of deduced amino acid sequences revealed all the isolates of *B. trigonocephalum* clustered in one clade with boot value more than 500 and other strongyles were in another clade..

As expected *B. trigonocephalum* are much closed associated with strongyles of same super family such as *A. caninum* and also with horse strongyles β -tubulin isotype 1 sequences. The analysis also suggested that β -tubulin isotype 1 gene sequences of *B. trigonocephalum* isolates have closely related with *Trichostrongyles* and other helminthes clustered separately.

The study is concluded that beta tubulin isotype-1 gene of *B. trigonocephalum* and other trichostrongyles are highly conserved. The information of beta tubulin isotype-1 gene of *B. trigonocephalum* provided an idea for development of molecular tools to diagnosis of benzimidazole resistance at the early stage in the country.

Fig.1 Anterior end of *B.trigonocephalum* showing well

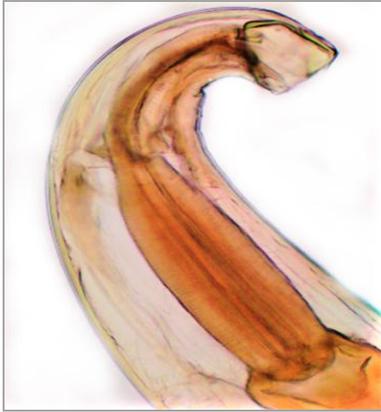


Fig.2 Posterior end of *B.trigonocephalum* showing spirally twisted



Fig.3 PCR amplification of β -tubulin gene of *B.trigonocephalum* Lane M: 1kb DNA ladder Lane 1&2: β - tubulin gene

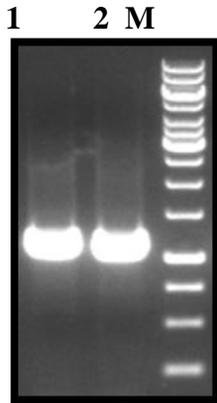


Fig.4 Colony PCR amplification of β -tubulin gene of *B.trigonocephalum* Lane M: 1kb DNA ladder Lane 1&2: β - tubulin gene

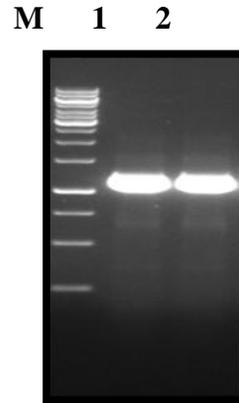


Fig.5 Release of insert from recombinant clone by *EcoRI* enzyme Lane M: 1kb DNA ladder Lane C1-C3: β - tubulin gene insert

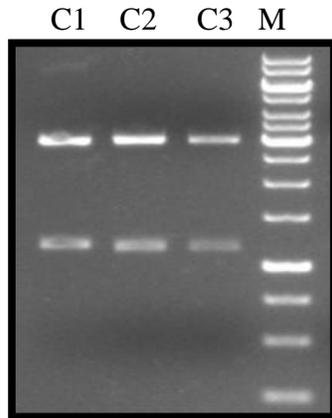
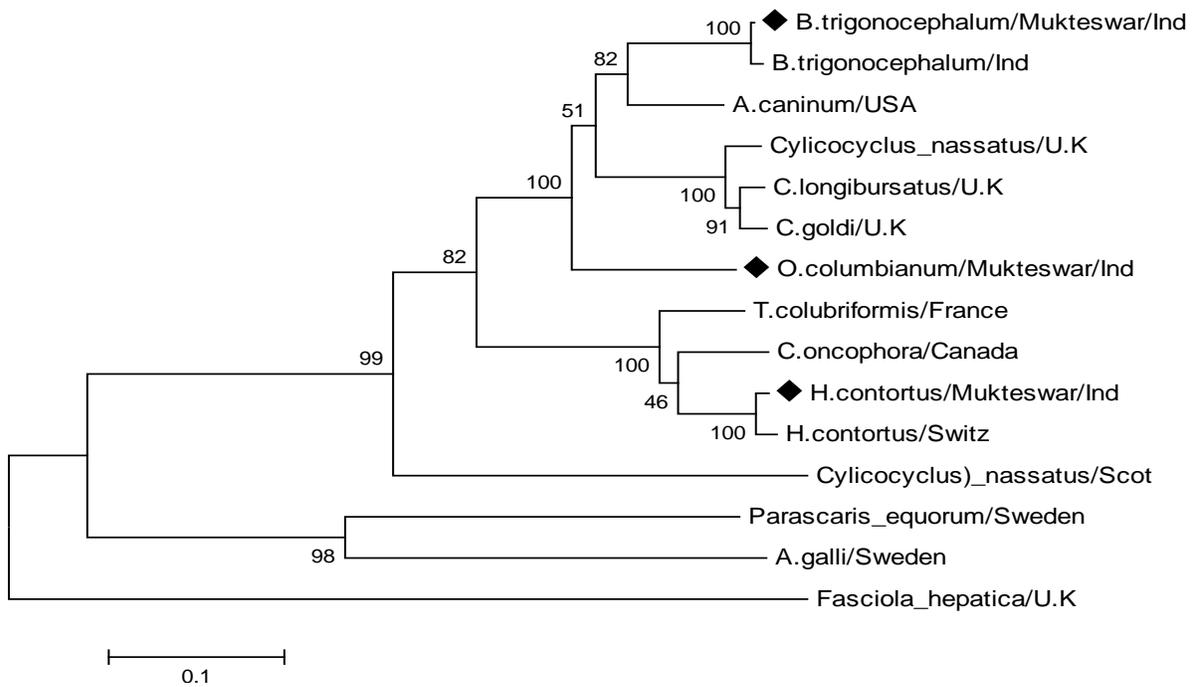


Fig.6 Pairwise relationship between *B. trigonocephalum* with other helminths

		Percent Identity																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		
Divergence	1	█	99.2	94.1	99.7	99.0	98.7	99.7	99.0	96.2	99.7	92.3	98.7	98.5	98.2	99.2	94.1	98.5	1	Mukteswar
	2	0.8	█	93.4	99.0	98.2	98.0	99.0	98.2	95.4	99.0	91.6	98.0	97.7	97.4	98.5	93.4	97.7	2	Mukteswar Full
	3	6.1	7.0	█	94.1	94.1	93.9	93.9	93.1	93.1	93.9	91.1	93.9	94.4	93.4	93.6	99.2	93.6	3	A galli Sweden
	4	0.3	1.0	6.1	█	99.0	98.7	99.5	98.7	96.2	99.5	92.1	98.7	98.5	98.2	99.2	94.1	98.5	4	A caninum USA
	5	1.0	1.8	6.1	1.0	█	99.7	98.7	98.0	97.2	98.7	91.6	99.7	99.5	99.2	98.5	94.1	99.5	5	C oncophora CAN
	6	1.3	2.1	6.4	1.3	0.3	█	98.5	97.7	96.9	98.5	91.3	99.5	99.2	99.0	98.2	93.9	99.2	6	C pectinata BLY
	7	0.3	1.0	6.4	0.5	1.3	1.5	█	98.7	95.9	99.7	92.1	98.5	98.2	98.0	99.0	93.9	98.2	7	Cst goldi UK
	8	0.3	1.0	6.4	0.5	1.3	1.6	0.5	█	95.2	98.7	91.8	98.2	97.4	97.2	98.2	93.1	98.0	8	Cst longibursatus UK
	9	3.2	4.0	6.4	3.2	2.1	2.4	3.4	3.4	█	95.9	91.1	96.9	96.7	96.4	95.7	93.1	96.7	9	Cyc nassatus SCOT
	10	0.0	0.8	6.1	0.3	1.0	1.3	0.0	0.3	3.2	█	92.1	98.5	98.2	98.0	99.0	93.9	98.2	10	Cyc nassatus UK
	11	8.1	8.9	9.5	8.4	8.9	9.2	8.4	7.9	8.7	8.1	█	91.8	91.1	90.8	91.6	91.6	91.6	11	F hepatica UK
	12	1.3	2.1	6.4	1.3	0.3	0.5	1.5	1.0	2.4	1.3	8.7	█	99.2	99.0	98.2	93.9	99.7	12	H contortus BLY
	13	1.5	2.3	5.8	1.5	0.5	0.8	1.8	1.8	2.6	1.6	9.5	0.8	█	98.7	98.0	94.4	99.0	13	H contortus MUK
	14	1.8	2.6	7.0	1.8	0.8	1.0	2.1	2.1	2.9	1.8	9.8	1.0	1.3	█	97.7	93.4	98.7	14	H contortus SWITZ
	15	0.8	1.5	6.7	0.8	1.5	1.8	1.0	1.0	3.7	0.8	8.9	1.8	2.1	2.3	█	93.6	98.0	15	O columbianum
	16	6.1	7.0	0.8	6.1	6.1	6.4	6.4	6.4	6.4	6.1	8.9	6.4	5.8	7.0	6.7	█	93.6	16	P equorum Sweden
	17	1.5	2.3	6.7	1.5	0.5	0.8	1.8	1.3	2.6	1.6	8.9	0.3	1.0	1.3	2.1	6.7	█	17	T colubriformis FRANCE
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17			

Fig.7 Molecular phylogenetic analysis by Maximum Likelihood method



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