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Molecular Characterization of β-Tubulin Isotype-1 Gene of *Trichostrongylus colubriformis*

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Benzimidazole resistance (BZR) is known to be associated with single nucleotide polymorphisms (SNPs) on beta-tubulin isotype 1 gene at any of F200Y, F167Y and E198A positions. Characterization of beta-tubulin isotype 1 gene is prerequisite for designing molecular diagnostic method for ascertaining BZR at the early stage of development. Present study was designed to characterize beta tubulin isotype-1 gene of *Trichostrongylus colubriformis* of small ruminants. Total RNA was extracted from male *T. colubriformis*. The β-tubulin gene complementary DNA (cDNA) was reverse transcribed from total RNA. The truncated β-tubulin gene of 1202 bp was PCR amplified from complementary DNA (cDNA), cloned in pGEM®-T easy TA cloning vector, custom sequenced and analysed. The analysis of deduced amino acid sequence revealed that the Uttarakhand isolate is susceptible to benzimidazole resistance as the amino acid on 200th position is phenylalanine (F200Y). The amino acid sequences showed 98.3% to 99% identity with the published *T. colubriformis* sequence as well as other trichstrongyle species. The phylogenetic analysis revealed that Uttarakhand isolate clustered with other trichostrongyles species. The information of beta tubulin isotype-1 gene of *T. colubriformis* provided an idea for development of molecular tools to diagnosis of benzimidazole resistance at the early stage.

Introduction

The nematode *Trichostrongylus colubriformis* (Order: Strongylida, Family: Trichostrongylidae), is amongst the most important gastrointestinal nematodes of small ruminants, and can be a major cause of economic losses to livestock farming. The

prevalence is ubiquitous and considered as second most important nematodes after *H. contortus* (O'Connor *et al.,* 2006). Adult *T. colubriformis* live in mucus-covered tunnels in the surface of the small intestine, where they feed on chyme components (Holmes, 1985). Heavy infections are associated with severe enteritis, characterized by extensive villus atrophy, mucosal thickening and erosion and infiltration of lymphocytes and neutrophils into affected mucosal areas (Holmes, 1985). Control of trichostrongylosis primarily has achieved by using broad spectrum chemotherapeutic agents like benzimidazoles (BZs), imidazothiazoles, tetrahydro-pyrimidines and macrocyclic lactones. Among broad-spectrum antihelminthics, BZs are most widely used against gastrointestinal nematode due to its high therapeutic index, absence of drug residue in milk and meat and economically viable (Humbert *et al.,* 2001). As a result of continued use, resistance to BZs has emerged world-wide within trichostrongylid parasitic nematodes including India (Gill, 1993, 1996; Waller, 1997; Silvestre and Humbert, 2000; Garg and Yadav, 2009; Swarnkar *et al.,* 1999, 2001; Chandra *et al.,* 2014, 2015). BZR in the three main gastrointestinal nematodes of sheep (*Teladorsagia circumcincta*, *T. vitrinus* and *Haemonchus contortus*) seems to be primarily linked to a point mutation at amino acid 200 of β-tubulin isotope-1 gene, which replaces a phenylalanine (Phe) with a tyrosine (Tyr) (Kwa *et al.,* 1993a, 1993b, 1994, 1995; Elard *et al.,* 1996, 1999). 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). Based on polymorphism between resistance and susceptible strains of *H. contortus*, several reports have described various methods for genotyping (Kwa *et al.,* 1994; Elard *et al.,* 1999; Silvestre and Humbert, 2000; Garg and Yadav, 2009; Chandra *et al.,* 2014, 2015). However, works on *Trichostrongylus* genus, particularly the most common *T. colubriformis* is meager. Therefore, it is necessary to characterize β –tubulin gene of *T. colubriformis* 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 *T. colubriformis*.

Materials and Methods

Study area and collection of parasites

Adult *Trichostrongylus colubriformis* isolate was collected from GI tract of goats slaughtered at local abattoir/meat shop at Mukteshwar (29°28'N and 79°39'E, 7500 feet above mean sea level), Uttrarakhand. Parasites were washed thoroughly in PBS (pH 7.4) and identified as per the morphological keys (Soulsby, 1982). The adult male worms were used either for direct extraction of RNA or preserved in RNA later RNA stabilization reagent at -20 ^{0}C for further use.

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from adult male *T. colubriformis* using QIAGEN RNeasy minikit (Qiagen, Germany) as per manufacturer's instructions. The complementary DNA (cDNA) was synthesized from the total RNA of adult male *T. colubriformis* using oligo dT primer and M-MLV reverse transcriptase (Fermentas, USA). PCR was standardized to amplify the truncated open reading frame of β-tubulin isotype-1 gene of *T. colubriformis.*

Polymerase chain reaction for amplification of truncated length β-tubulin gene

The primers were self-designed by using retrieved β-tubulin gene sequences of *T. colubriformis* (Acc. No. L23506) and

sequences of other strongyles (*Haemonchus contortus* Acc.no. EF198865.1, *Oesophagostomum columbianum,* Acc.no. KP792296.1, *Bunostomum trigonocephalum*, KP792295.1, *Cooperia pectinata*, EF198866.1) from NCBI GenBank.

The forward primer (5'GCC GGW CAR TGC GGH AAC CAG 3') and reverse primer (5'GTG AAY TCC ATT TCG TCC ATA C 3') were designed to amplify all the expected mutations for benzimidazole resistance present in the gene such as 125th, 167th, 198th and 200th position.

PCR was carried out in thin wall PCR tubes in 50 µl reaction volume. The PCR mixture consisted of cDNA as template, 15 pmoles of each primer (Forward and Reverse), 1.5 mM $MgCl_2$, 400 µM of each dNTPs, 10x pfu polymerase buffer and 2.5 IU of pfu polymerase (Fermentas, Germany). The reaction was standardized by initial denaturation at 95ºC for five minutes, followed by 35 cycles each at 95ºC for 30 sec, 57ºC for 30 sec and 72ºC for 75 seconds. The amplicons were electrophoresed using 1.2% agarose gel and the DNA migration and resolution pattern was examined by UV transillumination technique and the picture was documented by photography.

Cloning, sequencing and characterization of β-tubulin gene

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* Top10F competent cells by heat-shock method at 42^{o}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. Subsequently, the positive clone was inoculated in LB stab culture and custom sequenced.

Genetic characterization

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 *T. colubriformis* and other related *Trichostrongyle* species.

Results and Discussion

The adult *T. colubriformis* worms are small hair-like and fresh worms are brown in colour. Under light microscope, buccal capsule is absent; the excretory pore is opened near to anterior end and form a characteristic notch (Fig. 1). The males are 4-5.6mm long and females are 5-8mm. The spicules are stout equal, ridged and pigmented brown. There is a triangular prominence in distal part of spicule, which is form a characteristic stepped appearance (Fig. 2). Gubernaculum is present. In female, the eggs are arranged in uterus usually side by side (Fig. 3).

Amplification of truncated length β-tubulin gene sequence of *T. colubriformis*

The β-tubulin isotype-1 gene was amplified as a single, approximately 1202 bp fragment by polymerase chain reaction in agarose gel electrophoresis (Fig. 4). The PCR product was purified and the concentration of purified β-tubulin gene was 37 ng/µl. The ligated amplicon with pGEM®-T easy TA cloning vector was successfully transformed as evidenced by appearing colonies in the LB Amp+ X gal+ IPTG+ plates and by colony PCR (Fig. 5).

Fig.1 Anterior end of *Trichostrongylus colubriformis:* showing excretory notch

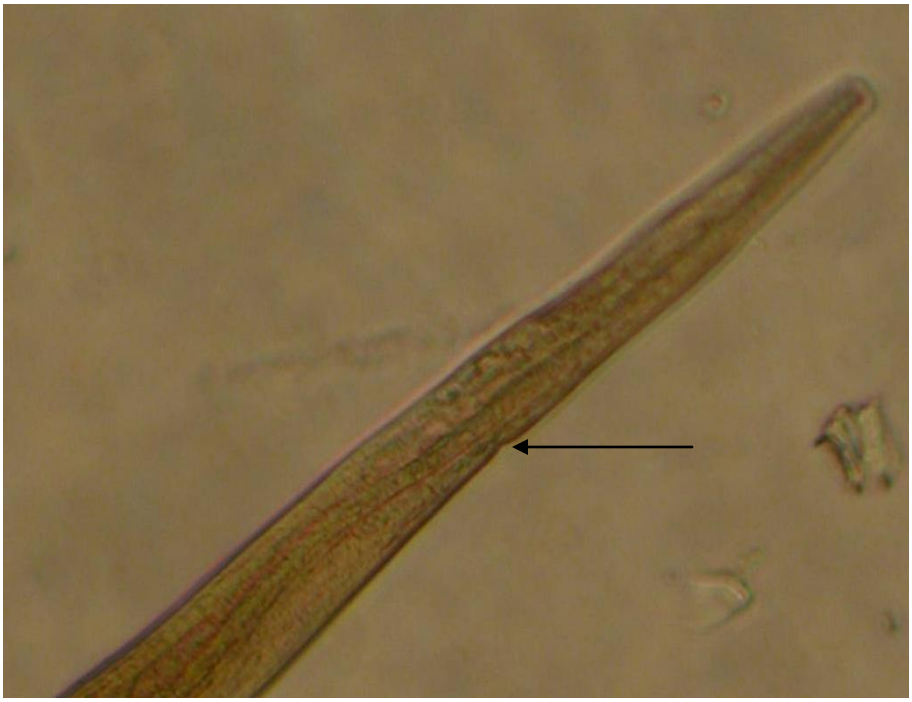
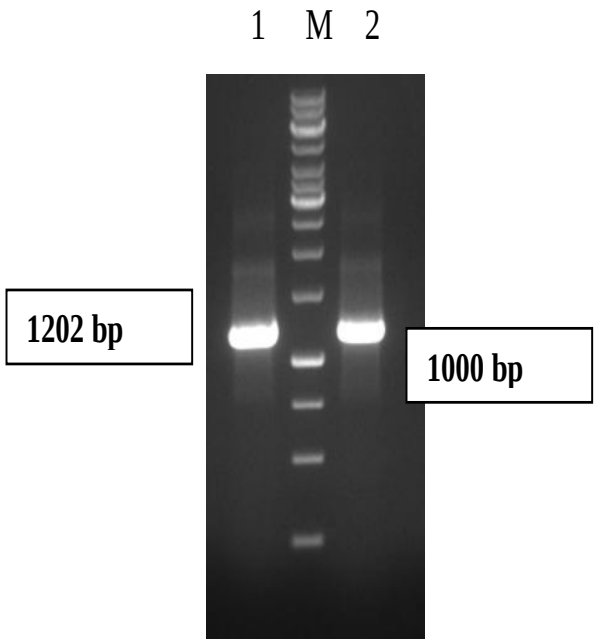


Fig.2 Spicules of *Trichostrongylus colubriformis*

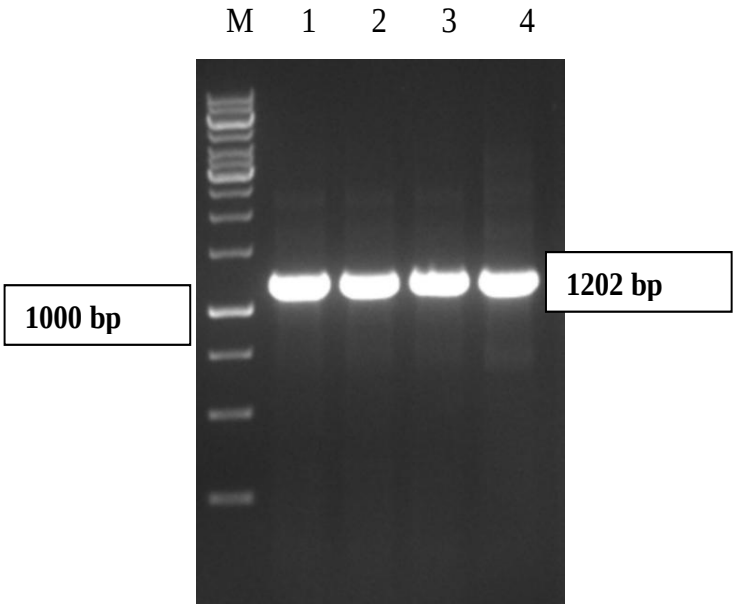
Fig.3 *Trichostrongylus colubriformis*- female showing arrangement of eggs in the uterus (occurred in side by side)

Fig.4 PCR amplification of truncated β-tubulin gene of *T. colubriformis*



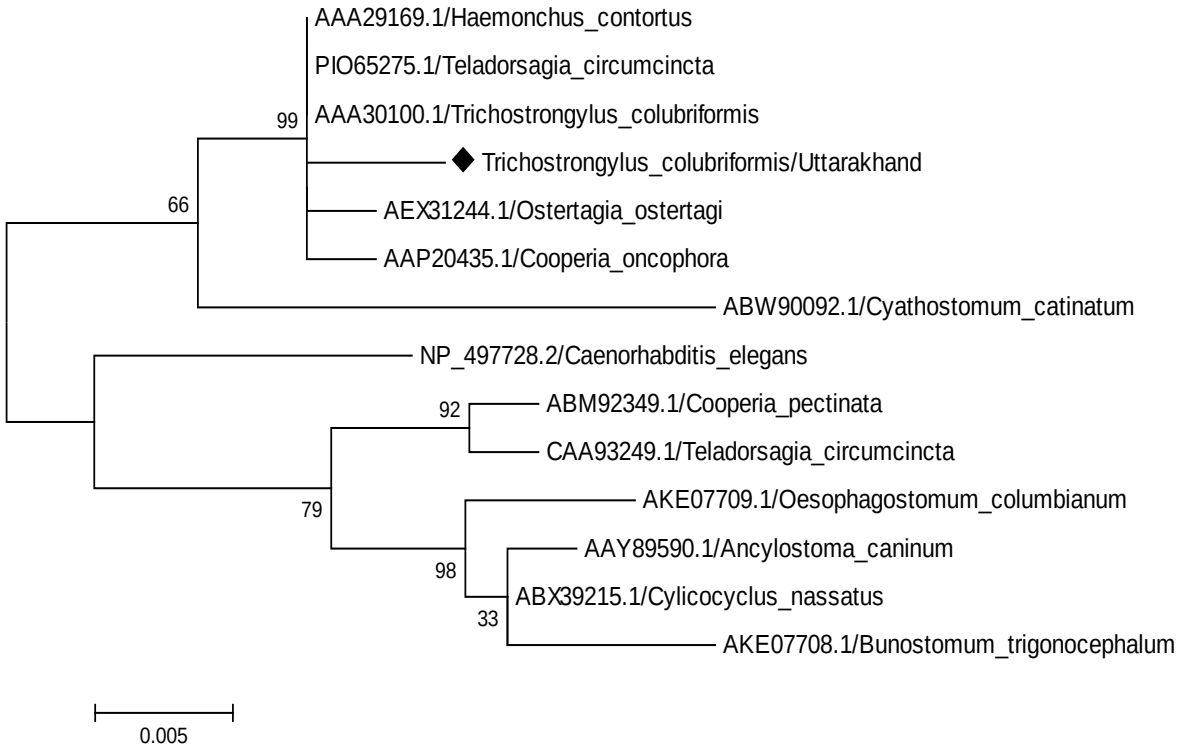
Lane M: 1kb DNA ladder (Thermo Scientific)
Lane 1 and 2: β-tubulin gene amplicons (1202bp)

Fig.5 Colony PCR product of β-tubulin gene of *T. colubriformis*



Lane M: 1kb DNA ladder (Thermo Scientific)
Lane 1-4: Colony PCR (1202bp)

Fig.6 Phylogenetic analysis for Uttarakhand isolates of *T. colubriformis* with published *T. colubriformis* and other strongyles



The positive clones harbouring β-tubulin gene was custom sequenced for nucleotides using T7 promoter forward and SP6 reverse primers by Sanger's dideoxy chain termination method.

The analysis of sequence revealed the size of the β-tubulin isotype- 1 gene was 1202bp as expected. The nucleotide sequences were deduced for obtaining amino acid sequence. The amino acid sequences showed 98.3% to 99% identity with the published *T. colubriformis* sequence as well as other strongyle species. The phylogenetic analysis on amino acid sequences revealed *T. colubriformis* and other trichostrongyles are clustered in one clade and other strongyles were in another clade (Fig. 6).

From Indian perspective, our understanding of the genesis of anthelmintics resistance is meager particularly in *T. colubriformis*. 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 present study, beta tubulin isotype-1 gene of adult male *T. colubriformis* isolate from Mukteshwar, Uttarakhand was characterised. To avoid eggs genome contamination in RNA isolation, male worm was used for characterization. The parasites were identified morphologically based on morphological keys (Soulsby, 1982; Taylor *et al.,* 2015). The spicules are specific in *T. colubriformis*, both spicules are equal in size with characteristic stepped appearance. But in *T. axei*, the spicules are dissimilar (Soulsby, 1982). In female, the eggs are arranged in uterus usually side by side when compared to another common *T. axei*, where eggs are arranged in pole to pole (Urquhart *et al.*, 1996).

The self-designed degenerative primers were used for amplification of beta tubulin isotype-1 and the size of the amplicons was 1202bp as expected. A degenerative primer sequence is that if some of its positions have several possible bases. The degeneracy of the primer is the number of unique sequence combinations it contains. The degeneracy will applicable when studying a family of genes that is known only in part, or is known in a related species (Linhart and Shamir, 2005). Here degenerative primers used due to non-amplification of specific primers. The primers were designed to amplify the all possible mutation sites such as 167th, 198th and 200th positions. Mutation at amino acid 200 of the beta tubulin isotype-1 (Phe to Tyr) is mostly responsible for resistance (Kwa *et al.,* 1994; Rufener *et al.,* 2009). Further, 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). However, except 200th position mutation, none of other changes were on reports in *T. colubriformis.* The characterization of beta tubulin isotype-1 revealed that the organism is susceptible to benzimidazole resistance as amino acid on 200th position is phenylalanine.

The deduced amino acid sequences were showed closer homology between Uttarakhand *T. colubriformis* with published sequence as well as with other trichostrongyles. Since the beta tubulin is one of the framework proteins of the cell function, many of the amino acids will be conserved. Therefore, less variation was observed across trichostrongyle species and single mutation may creates considerable functional consequences. As expected, phylogenetic tree on amino acid sequences revealed *T. colubriformis* and other trichostrongyles are clustered in one clade and other strongyles were in another clade.

The study is concluded that beta tubulin

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

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