

Variation in the β -tubulin isotype 1 gene at codon 167 and 200 responsible for benzimidazole resistance in *Haemonchus contortus* in sheep of Krishna District, AP, India

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ABSTRACT

A study was carried out for detection of single nucleotide polymorphisms at codon 167 and 200 of the β -tubulin isotype 1 gene that are linked to BZ resistance of *Haemonchus contortus* in sheep. A total of 226 adult male worms were genotyped from different regions of Krishna district, Andhra Pradesh. Amplification of DNA from each worm by PCR, followed by semi-nested PCR, yielded an expected fragment of 488 bp product. The PCR product was subsequently digested with restriction endonuclease *SnaBI* and *TaaI* to detect mutation at codon 167 and 200 respectively. On digestion, three different fragment patterns were observed at codon 200, one with 215 bp, 206 bp and 67 bp (homozygous resistant; 'rr'), the second with 282 bp, 215 bp, 206 bp and 67 bp (heterozygous; 'rS'), and another with 282 bp and 206 bp fragment (homozygous susceptible; 'SS'). No resistance allele (TAC) was evident at codon 167 in any worms including the worms that carried a susceptible allele (TTC) at codon 200. The overall genotype frequencies varied significantly ($P < 0.01$) with respect to the β -tubulin gene/*TaaI* locus in the study area. The frequency of 'rS' (64.60%) genotypes was high compared to that of 'rr' and 'SS' genotypes. The genotype frequency for 'rr' worms ranged from 6.25% to 31.82% among different regions. In general, the prevalence of BZ resistance allele was found to be significantly ($P < 0.01$) higher (54.0%). Results revealed β -tubulin isotype 1 polymorphism at codon 200 in *H. contortus* of sheep indicating the occurrence of resistance allele in the study area.

Key words: *Haemonchus contortus*; benzimidazole resistance; β -tubulin gene polymorphism; PCR-RFLP

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Introduction

Resistance to anthelmintics in gastrointestinal nematodes represents a major threat to sheep management and production. *Haemonchus contortus* is one of the pathogenic blood sucking nematode in small ruminants, and the major approach to control the rate of infection in endemic regions globally depends on chemotherapy using benzimidazoles (BZ). It is evident that BZ binds to β -tubulin and interferes with the polymerization of microtubules there by destabilizing the intracellular environment in the parasite. Frequent use of BZ for decades has forced the selection and emergence of BZ resistant *H. contortus* populations in several areas of the world. Genetic studies on BZ resistance by LE JAMBRE (1985) revealed that more than one genetic locus is involved in BZ resistance in *H. contortus*. One of the genetic mechanisms conferring resistance in field populations of *H. contortus* is a change of TTC to TAC at codon 200 (F200Y) of the β -tubulin isotype 1 gene (ELARD et al., 1999) which has been frequently reported. A similar polymorphism (TTC to TAC) at codon 167 (F167Y) is acknowledged to be involved in BZ resistance in *H. contortus*, but no simultaneous substitutions at two codons 167 and 200 have been observed in the same allelic sequence of *H. contortus* of small ruminants, so they are thought to be mutually exclusive (SILVESTRE and CABARET, 2002).

A clear appraisal of the resistance or susceptibility of the target worms is required for effective control strategies and also to maintain the efficacy of the currently available anthelmintics wherever resistance has not emerged. This is only possible if sufficient means are available for the diagnosis of anthelmintic resistance at an early stage. Conventional methods, viz. the faecal egg count reduction test (FERT), egg hatch assay (EHA) and larval development assay (LDA) can only help to detect resistance when 25 per cent of the individuals in the population are already resistant (MARTIN et al., 1989), and these tests are not economical. In contrast, PCR based assays can detect resistance at the initial stage, even when only one per cent of the resistant parasites are in the population (SILVESTRE and HUMBERT, 2000), and these assays are very useful in perception of the BZ resistance mechanism.

In India, the occurrence of BZ resistance in *H. contortus* has been reported in different sheep populations since 1976 by conventional (VARSHNEY and SINGH, 1976) and molecular techniques (TIWARI et al., 2006; KAUSHIK et al., 2016). Among the molecular assays, PCR-RFLP has been found to be an economically viable genotyping assay and to detect the SNP's at codon 167 and 200 of the β -tubulin isotype 1 gene of *H. contortus* (SHOKRANI et al., 2012). Owing to this and due to the lack of reports on BZ resistance in sheep from Andhra Pradesh, an attempt was made for the first time to investigate the resistance status of *H. contortus* in sheep populations in Krishna district, Andhra Pradesh, India.

Materials and methods

Collection of parasites (Haemonchus contortus): Adult male *Haemonchus contortus* worms were collected directly from the abomasum of various sheep brought to the local abattoirs in Krishna district, Andhra Pradesh. In order to have a representative sample of *H. contortus* worms from all the Animal Husbandry divisions of the district, a Random Stratified sampling method was used to select the areas, and the worms were collected from the Vijayawada (31), Gannavaram (60), Hanuman Junction (31), Ibrahimpatnam (32), Nuziveedu (23), Gudiwada (22) and Machilipatnam (27) regions (Fig. 1). A total of 226 parasites were collected and preserved in 70% alcohol at $-20\text{ }^{\circ}\text{C}$ until use, after washing in PBS (pH 7.4).

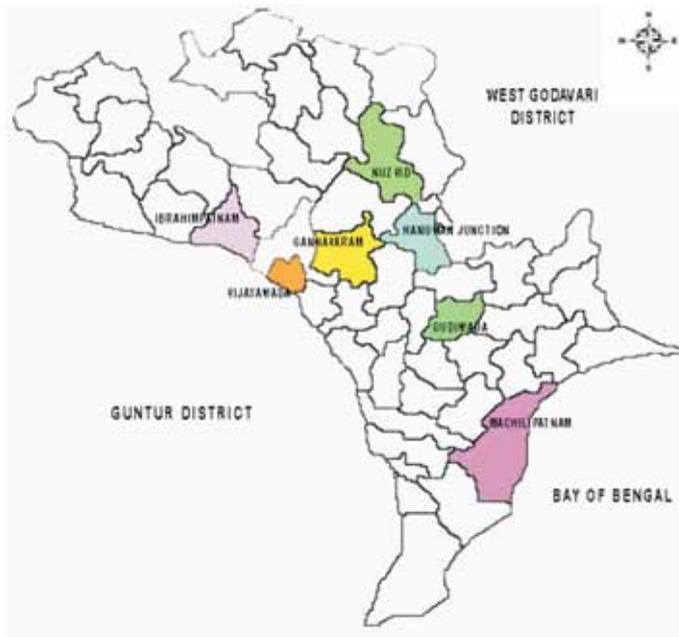


Fig. 1. Location map of sampling areas

Genotyping of β -tubulin gene by polymerase chain reaction (PCR) / RFLP

DNA extraction. The genomic DNA was isolated from each adult male *H. contortus* worm using the protocol explained by TIWARI et al. (2006), with minor modifications. Each individual *H. contortus* worm was digested in a digestion mixture comprised of 195 μL of lysis buffer (10 mM TrisHCl, 0.1 M EDTA, 2.0 per cent SDS, distilled water) and 10 μL Proteinase-k (20 mg/mL), at $37\text{ }^{\circ}\text{C}$ for 4 hours. After incubation, 200 μL of

phenol:chloroform:iso-amyl alcohol (25:24:1) was added, mixed and centrifuged at 12000 rpm for 5 minutes. The aqueous phase was transferred to a fresh 2 mL micro centrifuge tube. Finally, the DNA was precipitated by absolute isopropanol at 12000 rpm and the precipitated DNA was again dissolved in an equal volume of 70% alcohol and centrifuged at 13000 rpm to recover the DNA pellet. The dried DNA pellet was resuspended in 30 μ L of nuclease free water and stored at -20 °C until further use.

Oligonucleotide primers. The forward (5'-GGTTGTGATTGCCTTCAGGT-3') and reverse (5'-GATCAGCATTTCAGCTGTCCA-3') primers were custom designed for amplification of the β -tubulin isotype 1 gene of *H. contortus*, capturing the mutation site on the basis of the sequence available from the NCBI (GenBank Accession No. X80046.1). UT-HC167 MF-primer (modified forward primer) was adopted from already published primers (SHOKRANI et al., 2012) for semi-nested PCR. Primers were diluted in nuclease and protease free water to give a final concentration of 10 pmoles/ μ L.

PCR amplification and RFLP of the β -tubulin gene. Amplification of the β -tubulin isotype 1 gene from the genomic DNA of each worm was carried out in a Prima-Duo Thermal cycler (Himedia, India) in a final reaction volume of 15 μ L, using the forward and reverse primers. Volumes of 80 ng of extracted DNA sample and 10 pmoles of each forward and reverse primer were added to the mastermix. The mastermix (Eppendorf) for PCR reactions was with a final concentration of magnesium 1.5 mM, dNTPs 200 μ M and Taq polymerase 1.25 U. Amplification was performed with a program of 3 min at 94 °C followed by 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s and a final elongation step at 72 °C for 5 min. Semi-nested PCR was performed on the diluted (1:20) PCR product with UT-HC167 MF-primer, as per the protocol explained by SHOKRANI et al. (2012). Five microliters of PCR amplicons were analyzed on ethidium bromide stained 2% agarose gel, and the sizes and quantities of the PCR products were verified by comparison with a quantitative ladder.

The PCR products generated were digested separately with *TaaI* (Thermoscientific) for recognition of the nucleotide sequences 5'-ACNGT-3' at codon 200, and with *SnaBI* (Thermoscientific) for recognition of the nucleotide sequences 5'-TACGTA-3' at codon 167. The reaction was accomplished by digestion at 65 °C and 37 °C overnight for *TaaI* and *SnaBI* enzymes respectively. To control enzyme activity, the semi-nested PCR product without a restriction enzyme was used as a positive control, and a mix inoculated with ultrapure water in a substitution of PCR product and enzyme was used as a negative control. Digested products were separated on a 2.0% agarose gel to observe the polymorphic patterns and thus the genotypes of the β -tubulin gene of *H. contortus*.

Statistical analysis. The genotypes were detected by observing the RFLP pattern of each sample in agarose gels for the β -tubulin gene. The allele frequencies of each allele were calculated separately for the different codons 167 and 200. Genotype and allele frequencies were calculated as per FALCONER and MACKAY (2009). The chi-square

test of goodness of fit was carried out with the observed and the expected numbers to check whether the worm population was in Hardy - Weinberg equilibrium. F- statistics (WEIR and COKERHAM, 1984) were estimated for analysis of the population structure of the parasite with respect to their resistance to BZ.

Results

Amplification the β -tubulin isotype 1 gene. A total of 226 worms were genotyped for detection of point mutations in the β -tubulin isotype 1 gene of *H. contortus*. Amplification of DNA from each worm yielded the expected fragment of 629 bp product in primary PCR. Semi-nested PCR resulted an expected fragment of 488 bp product, which was used for further analysis. Oligonucleotide primers did not yield PCR product with the negative control.

*The β -tubulin isotype 1 gene/TaaI polymorphism in *H. contortus* in sheep.* On digestion of the PCR product with restriction endonuclease *TaaI* three different patterns were observed at codon 200, one with 215 bp, 206 bp and 67 bp, the second with 282 bp, 215 bp, 206 bp and 67 bp, and the third with 282 bp and 206 bp fragment (Fig. 2) which were referred to 'rr' (homozygous resistant; TAC/TAC), 'rS' (heterozygous; TAC/TTC) and 'SS' (homozygous susceptible; TTC/TTC) genotypes, respectively. In 'rs' and 'rr' patterns, by virtue of the very few base pair differences between the 215 and 206 bp bands, they were not wide enough apart to be appreciated as different bands on visualization with gel electrophoresis. These 215 and 206 bp bands (Fig. 2 lane: 4-8) can be differentiated from 206 bp (Fig. 2, lane: 1-3) with respect to intensity and the presence of an additional 282 bp band in 'rs' genotypes.



Fig. 2. RFLP Pattern of β -tubulin isotype1 gene/TaaI in *H. contortus*. M: 25 bp ladder, PC: Positive control, N: Negative control, Lane 1-3: SS (Susceptible), Lane 4-6: rr (Homozygous resistant), Lane 7-8: rS (Heterozygous).

Table 1. Genotypic and allelic frequencies of benzimidazole resistance in adult *H. contortus* from different regions of Krishna district, Andhra Pradesh

Region	Genotypic frequency			χ^2 value	Allele frequency	
	Homozygous resistant (rr)	Heterozygous (rS)	Homozygous susceptible (SS)		Resistant (r)	Susceptible (S)
Ibrahimpattam**	6.25 (2)	75.0 (24)	18.75 (6)	8.7	0.44	0.56
Vijayawada**	12.9 (4)	77.42 (24)	9.68 (3)	9.4	0.52	0.48
Nuziveedu	30.43 (7)	34.78 (8)	34.78 (8)	2.1	0.48	0.52
Hunuman Junction**	29.03 (9)	67.74 (21)	3.22 (1)	6.3	0.63	0.37
Gannavaram**	23.33 (14)	66.67 (40)	10.0 (6)	7.7	0.57	0.43
Gudiwada*	31.82 (7)	68.18 (15)	0	5.8	0.66	0.34
Machilipattam	18.52 (5)	51.85 (14)	29.63 (8)	0.07	0.44	0.56
Overall**	21.24 (48)	64.60 (146)	14.16 (32)	20.14	0.54	0.46

** Significant, $P < 0.01$, * Significant, $P < 0.05$, Values in parenthesis are the numbers of individuals

The genotyping results with regard to codon 200 of the β -tubulin isotype 1 gene of individual *H. contortus* collected from different regions of Krishna district are presented in the Table 1. Except for the worm populations collected from Nuziveedu and Machilipattam, the genotype frequencies were not in accordance with the Hardy-Weinberg equilibrium. The frequency of resistant genotypes varied from 6.25 to 31.82 per cent in the district. The frequencies of BZ resistant genotypes were less in Ibrahimpattam, Nuziveedu and Machilipattam areas, but the difference was only significant in Ibrahimpattam ($P < 0.01$). In those areas where the resistant genotype frequency was higher, the differences were observed to be statistically significant ($P < 0.01$). None of the worms collected from Gudiwada region were homozygous susceptible.

The overall genotype frequencies varied significantly ($P < 0.01$) with a high frequency of 'rS' (64.60%) genotypes compared to that of 'rr' (21.24%) and 'SS' (14.16%) genotypes. With regard to allelic frequency at codon 200, the frequency of the BZ resistant allele 'r' was found to be significantly ($P < 0.01$) higher (54.0%) as compared to the BZ susceptible allele 'S'. F-statistics analysis revealed that the F_{IS} and F_{ST} values were -0.33 and 0.02 respectively at the 200 codon.

β -tubulin isotype 1 gene/SnaBI polymorphism in H. contortus in sheep. On digestion of the PCR product with *SnaBI* all 226 samples were found to yield an uncut 488 bp fragment (Fig. 3), as there was no recognition site within the amplified sequence. All the worms which were monomorphic at codon 167 revealed susceptible alleles 'TTC/TTC' and no resistant alleles (TAC) were evident. F-statistics could not be performed at codon 167 as all the worms are monomorphic.

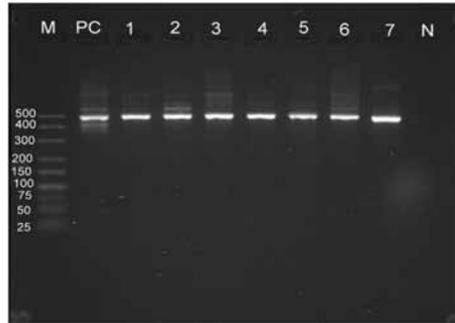


Fig. 3. RFLP Pattern of β -tubulin isotype1 gene/SnaB1 in *H. contortus*. M: 25 bp ladder, PC: Positive control, N: Negative control, Lane 1-7: Monomorphic pattern of different worms.

Discussion

The significant association between the SNPs at codon 167, 198 and 200 of the β -tubulin gene in conferring BZ resistance in *H. contortus* has been experimentally evidenced for a decade, and these mutations can be used as diagnostic tags to detect BZ resistance in *H. contortus*. The perusal of literature revealed isolated reports on the significance of SNP at codon 167 of the β -tubulin isotype 1 gene in *H. contortus*, particularly from India. In the present study, RFLP analysis of the β -tubulin gene in *H. contortus* revealed polymorphism at codon 200 and the absence of polymorphism at codon 167, agreeing with the report by SILVESTRE and HUMBERT (2002), where the presence of one SNP is not usually accompanied by a second. The polymorphism at codon 200 is the most frequent mutation conferring BZ resistance in *H. contortus* in small ruminants, and mutations at codon 167 have been observed to occur rarely in the field (SILVESTRE and CABARET, 2002), although this is the frequent and primary mutation in cyathostomins (HODGKINSON et al., 2008). Recent analysis of SNPs in *H. contortus* populations in Ceará State, Brazil by SANTOS et al. (2017) revealed a higher frequency of resistant allele at SNP F167Y than those at F200Y. Resistant allele were not observed at codon 167 even in the worms that carried susceptible genotypes at codon 200, indicating that mutations at this codon are not common and thus do not contribute to BZ resistance in the study area. However, HODGKINSON et al. (2008) observed heterozygous genotypes at codon 167 and 200 in cyathostomins, and hypothesized that if mutations in the two alleles appeared at both codons in a parasite a stronger level of resistance would develop, leading to death. Further, CHAUDARY et al. (2015) reported the absence of SNP at both F167Y and F200Y in the β -tubulin isotype 1 gene of *H. contortus* collected from two sheep in one of the locations of the present study (Vijayawada), in order to establish the phylogenetic relationships of the gene.

Following the reports of HERLICH et al. (1981) and KWA et al. (1995), the heterozygotes in the present study were considered as susceptible since the resistant allele is recessive. The genotype frequencies of rr, rS and SS varied significantly ($P < 0.01$) in the study area, with a high frequency of BZ susceptible genotypes of *H. contortus* (78.76%) worms ('SS' and 'rS') carrying the TTC allele. Moreover, only homozygous ('rr') genotypes appeared to survive with the recommended dose of treatment (ELARD et al., 1999; HUMBERT et al., 2001). In contrast, TIWARI et al. (2006) reported the high frequency of resistant genotypes in *H. contortus* collected from different regions of Rajasthan, considering 'rS' as resistant genotypes.

The high frequency of susceptible genotypes in the present study might be due to the following reasons: The Krishna district is a region in Andhra Pradesh where agricultural practices are actively followed such as the practice of letting the sheep flock onto harvested fields. The practice of deep ploughing in the fields at regular intervals exposes the larvae to extreme environmental conditions which are adverse for continuation of infective stages and the perpetuation of parasites, resulting in low levels of infection and hence, low use of BZ and less selection pressure on resistant population of *H. contortus*. In addition, the practice of annual deworming and use of macrocyclic lactones and imidazothiazoles as alternatives to BZ in sheep could have caused the low incidence of BZ resistance in the flocks. Another possibility could be the parasite collection in summer and winter seasons, when treatment strategies are not practiced routinely. SINGH et al. (1999) opined that the incidence of resistance in parasites could be greater in those collected during the monsoon season when drenching practices in the flocks are highest.

The possibility of a high prevalence (64.60%) of 'rS' individuals might be due to the presence of the recessive allele 'r' at moderate frequency which results in more heterozygotes due to random mating. Another reason could be the use of under or over dosing of anthelmintics. A sub-therapeutic dose allows the survival of heterozygous worms (EGERTON et al., 1988) and was considered a crucial factor for the further development of resistant strains (SILVESTRE et al., 2001). Correspondingly, the increasing dosage of BZ reduced the 'SS' individuals and increased the TAC allele frequency, thereby increasing the number of 'rS' and 'rr' individuals in cyathostomes (PAPE et al., 2003). In accordance with the present findings, the frequency of 'rS' genotypes was high compared to that of 'rr' and 'SS' types in *H. contortus* in goats in Madhura district, India (KAUSHIK et al., 2016).

Although there were more overall susceptible genotypes in *H. contortus* of sheep in the study area, the resistant allelic frequency is significantly high ($P < 0.01$) due to the presence of a higher number of heterozygotes in *H. contortus* as they harbor both the alleles. In contrast, a high frequency of susceptible allele in *H. contortus* populations in sheep was reported by AMRUTHA (2015) elsewhere in the state. Resistant allele at

codon 200 on the β -tubulin gene in *H. contortus* of sheep in a moderate frequency and with a high percentage of heterozygotes in the study area warrants periodic investigations for the early detection of mutated alleles in nematode populations, since reversal to susceptible is not possible even using different anthelmintic molecules with no cross-resistance (LEIGNEL et al., 2010).

In conclusion, the present study supported the hypothesis that SNP at codon 200 of the β -tubulin isotype 1 gene is important in conferring BZ resistance in trichostrongylid nematodes, and another SNP at codon 167 of the β -tubulin gene is infrequent. Further periodical screening for resistant allele frequency is warranted to revisit deworming policies.

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Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial relationships that could be construed as a potential conflict of interest.

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SAŽETAK

Provedeno je istraživanje s ciljem otkrivanja polimorfizama pojedinačnih nukleotida u kodonu 167 i 200, β -tubulin izotip 1 gena koji je povezan s otpornošću ovčjeg parazita *Haemonchus contortus* prema benzimidazolu (BZ). Genotipizirano je ukupno je 226 odraslih muških crva iz različitih područja distrikta Krishna, Andhra Pradesh. Umnažanje DNA iz svakoga crva pomoću PCR-a praćeno je poluugniježdenim PCR-om, čime je proizveden očekivani fragment od 488 bp. PCR produkt potom je pomiješan s restriksijskim endonukleazama *SnaBI* i *TaqI* radi otkrivanja mutacije u kodonu 167 i 200. U kodonu 200 opažena su tri različita fragmenta, jedan s 215 bp, 206 bp i 67 bp (homozigotno rezistentan, „rr“), drugi s 282 bp, 215 bp, 206 bp i 67 bp (heterozigot, „rS“) i treći s 282 bp i 206 bp fragmentom (homozigotno sumnjiv, „SS“). Rezistentni alel (TAC) nije ustanovljen u kodonu 167 svih istraženih crva, uključujući crve koji su nosili sumnjivi alel (TTC) u kodonu 200. Ukupna učestalost genotipova znatno je varirala ($P < 0,01$) u odnosu na β -tubulin gen/*TaqI* lokus istraženog područja. Učestalost genotipa „rS“ (64,60 %) bila je visoka u usporedbi s učestalošću genotipova „rr“ i „SS“. Učestalost genotipa „rr“ kod crva iz različitih područja kretala se u rasponu od 6,25 % do 31,82 %. Općenito, opažena je signifikantno ($P < 0,01$) viša (54,0 %) prevalencija BZ rezistentnog alela. Rezultati potvrđuju da polimorfizam kodona 200 u β -tubulin izotip 1 genu ovčjeg parazita *H. contortus* upućuje na pojavu rezistentnih alela u istraženim područjima.

Ključne riječi: *Haemonchus contortus*; rezistencija na benzimidazol; polimorfizam gena β -tubulin; PCR-RFLP
