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Research Article

STRUCTURAL DIVERSITY IN BETA-TUBULIN ISOTYPE 1 PROTEIN OF BENZIMIDAZOLE RESISTANT AND SUSCEPTIBLE *HAEMONCHUS CONTORTUS*

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ABSTRACT: Reports of benzimidazole resistance across the globe are available. The resistance of the parasite to the benzimidazole group is due to the single nucleotide polymorphisms at three positions, 200, 198, and 167 of the β-tubulin isotype 1 gene. The mutation at position 200 is commonly reported in various parts of India. The present study analyzed the genetic diversity in ten isolates of *Haemonchus contortus* β-tubulin isotype 1 gene in different states of India. *In silico* models of susceptible and F200Y mutated β-tubulin were built and docking with albendazole was done. The result of the docking study showed the alteration in the conformation of the albendazole binding pocket of F200Y mutated β-tubulin in comparison to the susceptible β-tubulin model.

Keywords: β-tubulin, Benzimidazole resistance, *Haemonchus contortus*, Molecular docking, Mutation.

INTRODUCTION

Resistance of various parasites to different anti-parasitic drugs by the parasites infecting sheep is not a contemporary problem but recorded more in recent times [1].

In 1964, Drudge and co-workers first documented thiabendazole resistance [2]. Thereafter, several authors reported benzimidazole resistance from various parts of the world. At the molecular level, resistance to benzimidazole is linked primarily to the mutation in three amino acids at position F167Y, E198A, and F200Y in the gene β-tubulin isotype 1 [3, 4, 5].

The mutations in the β-tubulin gene were reported in *Teladorsagia circumcincta* [5, 6], *Ascaris lumbricoides* at position 167 [7], and 200 [8] from Brazil, *Haemonchus contortus* at position 198 and 200 from India [9, 10, 11] and at position 198 and 200 in China [12], *Bunostomum trigonocephalum* at position 200 [13], *Trichuris trichiura* and *Necator americanus* from Southern Mozambique [14].

Mutation at these positions alters the binding pocket formed by the β-tubulin for the benzimidazole drugs, thus reducing the binding affinity of the drug [15,16]. Robinson *et al.* (2004) gave a possible explanation of the role of phenylalanine residue at position 200 interacting with albendazole based on the molecular docking study [17]. The substitution of tyrosine for phenylalanine at position 200 shuts the hydrophobic binding pocket and thus hinders the entry of benzimidazole into the binding pocket. Molecular simulation studies showed that the binding of benzimidazole is also impaired by the mutation of amino acids at positions 198 in addition to 200, while the F167Y mutation has no discernible impact [18]. However, insignificant changes in the binding pocket of 167, 198, and 200 mutant β-tubulin models to the benzimidazoles were exemplified [19].

In the present study, ten isolates of β-tubulin isotype 1 of *H. contortus* from different parts of India were characterized to identify the resistance-associated mutation. Also, *in silico* molecular docking was performed to find the binding of albendazole to the susceptible and resistant *H. contortus* β-tubulin models.

MATERIALS AND METHODS

Collection of parasites

Adult *H. contortus* parasites were collected from the abomasum of sheep and goats in eight states of India - Assam, Gujarat, Kashmir, Madhya Pradesh, Tamil Nadu, Uttarakhand, Uttar Pradesh, and West Bengal. The latitude and longitude of the areas from where the adult worms were collected are presented in Table 1. The worms collected were preserved in RNA later and transported to the laboratory for further processing.

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Isolation of RNA and cDNA synthesis

The total RNA was extracted from the individual adult worms of each state using an RNease mini kit (Qiagen, Germany) following the protocol described. The isolated RNA (38- 149 ng/µL) was converted into complementary DNA using iScript™ cDNA Synthesis Kit (Bio-Rad).

PCR amplification of *H. contortus* β-tubulin isotype 1

The *H. contortus* β-tubulin isotype 1 was PCR amplified using the self-designed degenerated primers (forward primer 5'- ATG CGT GAA ATC GTT CAT GTG C - 3' and reverse primer 5'- TTA CTC CTC GGG RTA YGS CTC- 3'). The PCR reaction mixture was prepared in 25 µL of 10X $MgSO_4$ buffer, 200 µ mol dNTP, 10 pmol of forward and reverse primers, and 5 IU of Pfu polymerase. The PCR components were allowed to initially denature at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, and the annealing temperature was set to 59°C for 30 sec, and extension was done at 72°C for 55 sec, and the final extension for 10 min at 72°C. The amplified gene was visualized in 1% ethidium bromide-stained gel.

Cloning of *H. contortus* β-tubulin isotype 1

The amplified gene was purified from the gel using the QIAquick Gel extraction kit (Qiagen, Germany) and cloned into pGEM®-T easy TA cloning vector (Promega) following the manufacturer's protocol. The clones were screened by blue-white screening for the positive recombinant clones [20]. The recombinant clones were then custom-sequenced in both directions.

Analysis of the sequence

The amino sequence was deduced from the nucleotide sequence and the protein sequences were aligned using Clustal w in MEGA version 11.0.10 and analyzed for the single nucleotide polymorphism at positions 167, 198, and 200.

Homology modeling of β-tubulin isotype 1

Based on the result of the sequence analysis, the three-dimensional models of the susceptible and resistant β-tubulin were built in the HHPred server [21, 22, 23]. The obtained models were refined in the Deep Refiner server [24]. The refined models were then validated in SAVESv.6.0 server (https://saves.mbi.ucla.edu/). The refined model was used for *in-silico* molecular docking.

***In silico* molecular docking of β-tubulin isotype 1 and albendazole**

The albendazole structure was obtained from Pubchem and the protein data bank format was converted using OpenBabel version 3.1.1 [25]. The protein models and albendazole were prepared for docking using the AutoDock Tools (ADT) in MGL tools 1.5.6 [26]. The binding pocket of the protein model was determined in the CASTp 3.0 server [27]. The site-specific docking was performed in AutoDock 4.2.6 software. The docking results were visualized in UCSF Chimera 1.16 [28]. The interaction of the protein models and albendazole was analyzed in the Protein-Ligand Interaction Profiler (PLIP) server [29].

RESULTS AND DISCUSSION

The sequences of β-tubulin isotype 1 gene of ten sequences were obtained, viz. Guwahati, Assam (1), Valsad, Gujarat (1), Srinagar, Kashmir (1), Rewa, Madhya Pradesh (1), Tirunelveli, Tamil Nadu (1), Mukteshwar, Uttarakhand (2), Bareilly, Uttar Pradesh (2), and Kolkata, West Bengal (1) and were aligned and analyzed for identifying mutation. SNP was noticed only at position 200 (phenylalanine to tyrosine) in three isolates, one each from Tamil Nadu, Uttarakhand (Mukteshwar), and Uttar Pradesh (Bareilly) (Fig. 1). No mutations were detected at positions 167 and 198. The results show that SNP at 200 is more prevalent than the other two SNPs in India. The result of the present study is in accordance with the previous reports of Mohanraj *et al.* (2017) and other researchers, who reported mutation at codon 200 of β-tubulin by allele-specific PCR in *H. contortus* of Tamil Nadu and Uttar Pradesh [9, 10, 30]. *H. contortus*, *Bunostomum trigonocephalum*, *T. circumcincta*, and *Trichostrongylus colubriformis* β-tubulin isotype 1 of small ruminants of Uttarakhand showed a prevalence of SNP at position 200 of β-tubulin isotype 1 by allele-specific PCR [31, 32, 33].

For the *in-silico* study, the susceptible and F200Y mutant β-tubulin isotype 1 of *H. contortus* was homology modeled in the HHPred server using human gamma-tubulin bound to GDP (3CB2_A), Plinabulin Binding to two Tubulin Isotypes (6S8K_B) and BtubAB heterodimer structure from *Prosthecobacter dejongeii* (2BTQ_B) as the templates with the sequence identity of 23.8%, 24.3%, and 22.2%, respectively. The model was then refined in the DeepRefiner server and validated in the SAVES v.6 server, which showed an overall quality factor of 98.38 and 99.07% in the ERRAT server and 99.10 and 97.3% in the Verify 3D

Table 1. Latitude and longitude of the areas of the sample collection.

Areas of sample collection	Latitude	Longitude
Guwahati, Assam	26.1158° N	91.7086° E
Valsad, Gujarat	20.5992° N	72.9342° E
Srinagar, Kashmir	34.0837° N	74.7973° E
Rewa, Madhya Pradesh	24.5362° N	81.3037° E
Tirunelveli, Tamil Nadu	08.7139° N	77.7567° E
Mukteshwar, Uttarakhand	29.4604° N	79.6558° E
Bareilly, Uttar Pradesh	28.3670° N	79.4304° E
Kolkata, West Bengal	22.5726° N	88.3639° E

for susceptible and F200Y mutant β-tubulin models, respectively. In the Ramachandran plot, 92.7 and 90.6% of residues were in the most favored region and none were in the disallowed region for susceptible and F200Y mutant β-tubulin models, respectively (Fig. 2). The active site residues were predicted in the CASTp 3.0 server, as shown in Table 2.

The site-specific docking of susceptible and F200Y mutant β-tubulin models with albendazole was performed, and the lowest binding energy obtained was considered the best pose. The binding energy for the susceptible β-tubulin-albendazole docking is -8.51 kcal/mol, whereas -7.09 kcal/mol for the F200Y mutant

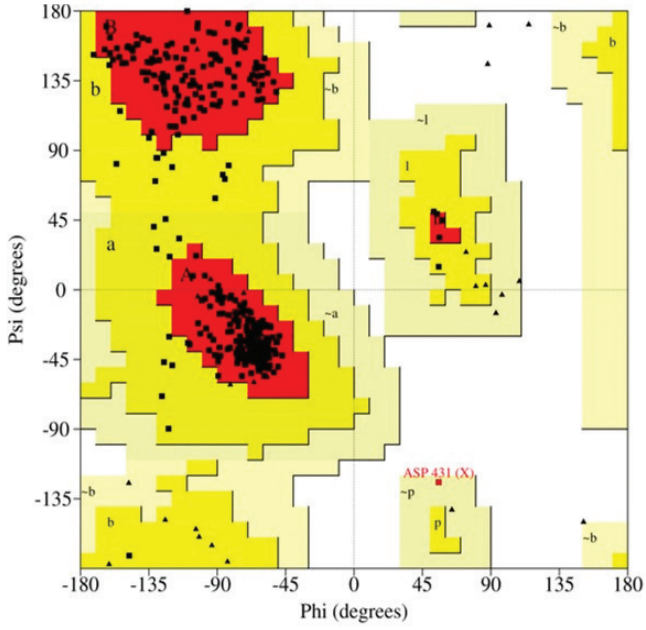
Table 2. The active site residues, area, and volume of the binding pocket of the susceptible and F200Y mutant β-tubulin β-tubulin isotype 1 models predicted in the CASTp server.

β-tubulin models	Area	Volume	Residues in the binding pocket
Susceptible	678.913	1269.941	4 ILE, 50 TYR, 134 GLN, 165 ALA, 167 PHE, 198 GLU, 200 PHE, 236 ILE, 237 THR, 239 CYS, 240 LEU, 246 LEU, 250 LEU, 252 LYS, 253 LEU, 255 VAL, 256 ASN, 257 MET, 258 VAL, 259 PRO, 260 PHE, 266 PHE, 312 THR, 313 VAL, 314 ALA, 315 ALA, 316 MET, 344 TRP, 345 ILE, 346 PRO, 347 ASN, 348 ASN, 350 LYS, 351 THR, 352 ALA, 368 ILE, 424 GLN, 425 TYR, 428 ALA, 431 ASP, 432 ASP, 433 MET, 434 GLY, 435 ASP, 437 ASP, 438 ALA, 439 GLU, 440 GLY, 441 GLY, 442 GLU, 443 GLU, 444 ALA
F200Y mutant	401.982	181.970	4 ILE, 133 PHE, 134 GLN, 163 ILE, 165 ALA, 167 PHE, 198 GLU, 200 TYR, 235 GLY,236 VAL, 237 THR, 238 THR, 239 CYS, 240 LEU, 246 LEU, 248 ALA, 250 LEU, 252 LYS, 253 LEU, 254 ALA, 256 ASN, 257 MET, 266 PHE, 312 THR, 313 VAL, 314 ALA, 315 ALA, 316 MET, 345 ILE, 346 PRO, 347 ASN,348 ASN, 350 LYS, 351 THR, 352 ALA, 366 THR, 368 ILE

Table 3. The residues of the susceptible and F200Y mutant β-tubulin isotype 1 models interacting with albendazole, hydrogen bonds, and hydrophobic bonds analyzed in PLIP server are presented.

β-tubulin models	Interacting residues	Hydrogen bonds	Hydrophobic bonds
Susceptible	50 TYR, 134 GLN, 165 ALA, 167 PHE, 198 GLU, 200 PHE, 236 ILE, 237 THR, 239 CYS, 240 LEU, 250 LEU, 253 LEU, 256 ASN, 257 MET, 266 PHE, 314 ALA, 315 ALA, 316 MET, 350 LYS, 352 ALA, 366 THR, 368 ILE	2 [198 GLU: 3.92, 2.73]	3 [253 LEU, 314 ALA, 368 ILE]
F200Y mutant	134 GLN, 165 ALA, 167 PHE, 198 GLU, 200 TYR, 234 SER, 235 GLY ,236 VAL, 238 THR, 239 CYS, 246 LEU, 250 LEU, 253 LEU, 254 ALA, 257 MET, 314 ALA, 315 ALA, 316 MET, 350 LYS, 352 ALA, 366 THR, 368 ILE	2 [315 ALA: 3.64; 368 ILE: 3.68]	7 [246 LEU (2), 256 ASN, 314 ALA, 350 LYS (2), 352 ALA]

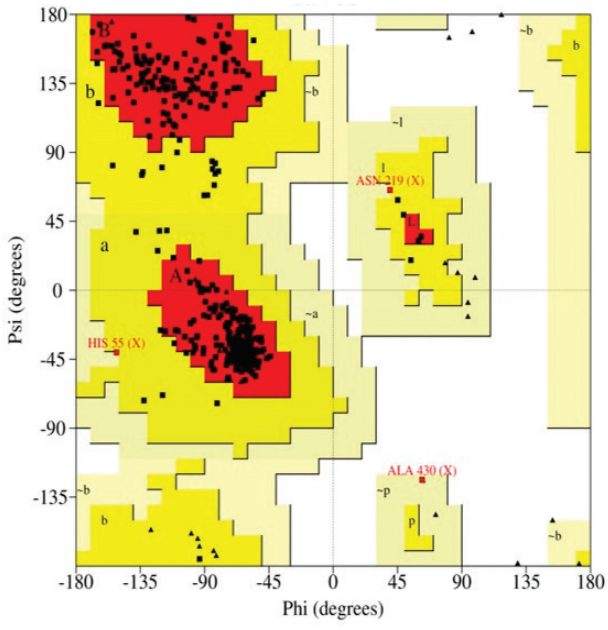
Fig. 1. The protein sequences of *H. contortus* β-tubulin isotype 1 isolate are aligned in MEGA 11.0.10 to identify the SNPs of amino acids at positions 167, 198 and 200. [The amino acid substitution was noticed at position 200 (F to Y) in isolates obtained from Bareilly (Uttar Pradesh), Mukteshwar (Uttarakhand) and Tamil Nadu].



Plot statistics

Residues in most favoured regions [A,B,L]	357	92.7%
Residues in additional allowed regions [a,b,l,p]	27	7.0%
Residues in generously allowed regions [~a,~b,~l,~p]	1	0.3%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	385	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	38	
Number of proline residues	19	
Total number of residues	444	

A



Plot statistics

Residues in most favoured regions [A,B,L]	349	90.6%
Residues in additional allowed regions [a,b,l,p]	33	8.6%
Residues in generously allowed regions [~a,~b,~l,~p]	3	0.8%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	385	100.0%
Number of end-residues (excl. Gly and Pro)	2	
Number of glycine residues (shown as triangles)	38	
Number of proline residues	19	
Total number of residues	444	

B

Fig. 2. Ramachandran plot showing the residues present in the most favored region; A) *H. contortus* susceptible β-tubulin isotype; 1 B) F200Y mutant β-tubulin isotype 1.

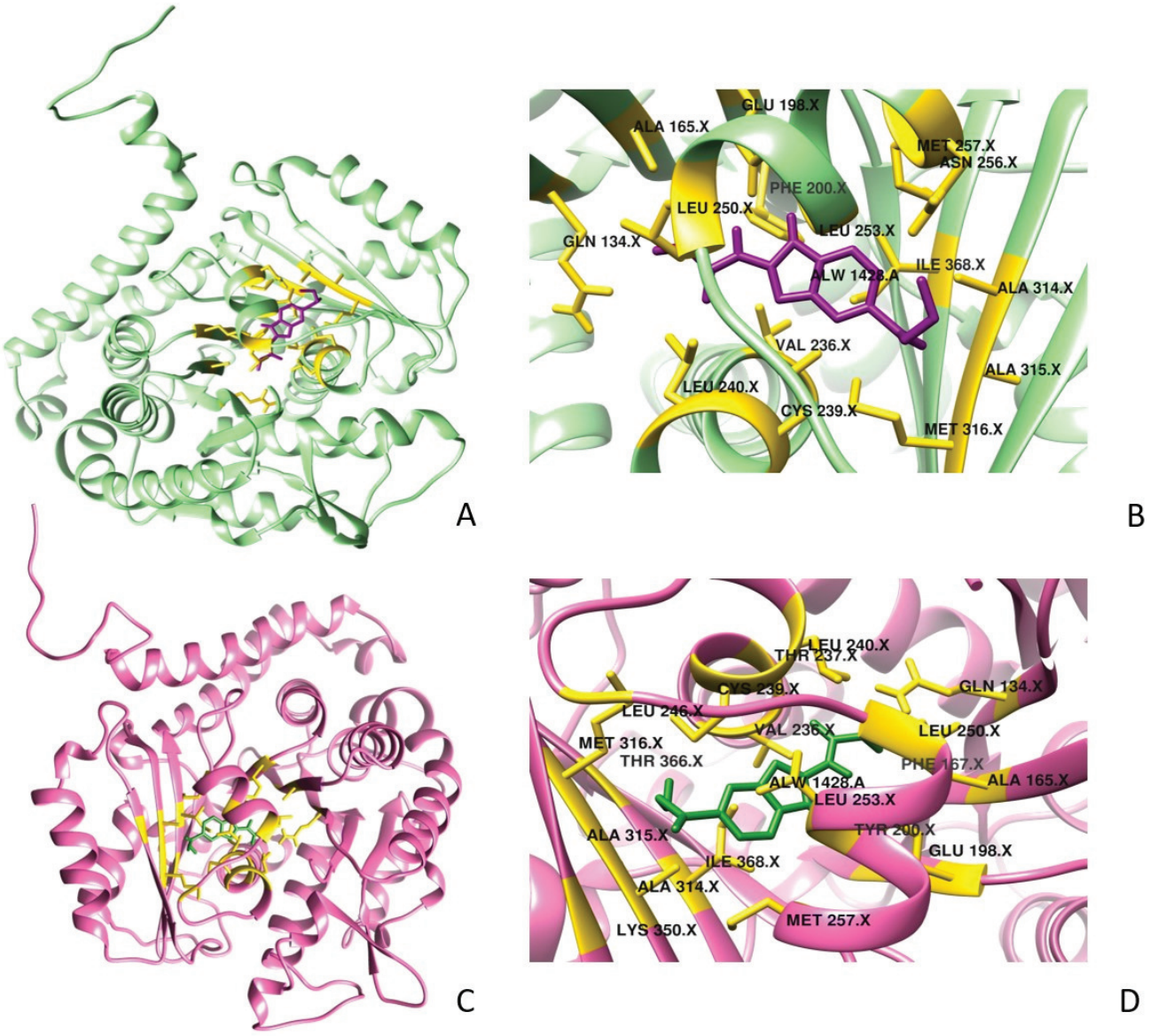


Fig. 3. The *H. contortus* susceptible (A) and F200Y mutant (B) β-tubulin isotype 1 interacting with albendazole and their interacting residues (C, D) visualized in UCSF Chimera are shown.

β-tubulin-albendazole docking. Robinson *et al.* (2004) performed a molecular docking study and reported that tyrosine amino acid is substituted for phenylalanine at position 200 closing the hydrophobic binding pocket and preventing albendazole from entering [17].

The interacting residues of the docking are given in Table 3. The docked complex and the interaction residues were visualized in the UCSF chimera and the depiction is shown in Fig. 3. The hydrogen bonds and hydrophobic bonds of the models formed with albendazole were analyzed in the PLIP interface. The susceptible β-tubulin formed two hydrogen bonds with residue 198 GLU of albendazole, whereas F200Y mutant β-tubulin formed two hydrogen bonds with 315 ALA and 368 ILE, which suggests the altered conformation of the active site.

The result shows that the binding stability of the albendazole is reduced in the F200Y mutant β-tubulin model.

CONCLUSION

The present study reports an F200Y mutation in β-tubulin isotype 1 of *H. contortus* from Tamil Nadu, Uttarakhand, and Uttar Pradesh. Other mutations were

not detected. *In silico* study suggests that mutation of amino acid at position 200 causes disturbances to the conformation of the active site and affects the stability of the albendazole within the active site. However, parasite β-tubulin is a promising drug candidate and needs alternate drugs to target and combat the benzimidazole-resistant parasites. In recent decades, computer-aided drug discovery has gained more attention as it reduces the time spent in the drug discovery process. Therefore, finding an alternative drug would be a wise decision to overcome parasitic resistance.

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