

Original Article

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# Resistance to benzimidazole in South India: Polymorphic variants in the β-tubulin gene of *Ascaris lumbricoides* and *Necator americanus*

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## ABSTRACT

**Objective:** Benzimidazole resistance in helminths is linked to single nucleotide polymorphisms (SNPs) at codons 167, 198, and 200. We studied the prevalence of these SNPs in the β-tubulin gene of *Ascaris (A.) lumbricoides* and *Necator (N.) americanus* so as to explore the resistance to benzimidazoles in South India.

**Methods:** DNA extracts of stool samples positive for *A. lumbricoides* and *N. americanus* were used for identification of SNPs. We utilized 22 samples to examine codon 167 SNPs and 15 samples for codon 198 and 200 SNPs in *N. americanus*. Three samples were used for SNP analysis of *A. lumbricoides*. Two conventional PCR assays were standardised for *N. americanus* (one targeting SNPs at coden 167 and another for 198 and 200), covering three SNPs, while a single PCR was developed for *A. lumbricoides*. The PCR amplified products were sequenced and compared with published sequences in NCBI for SNP identification.

**Results:** A total of 650 stool samples were collected from pregnant women and screened for the presence of *A. lumbricoides* and *N. americanus*, out of which 22 positive samples were used to examine codon 167 SNPs and 15 samples for codon 198 and 200 SNPs in *N. americanus*, and three samples were used for SNP analysis of *A. lumbricoides*. Despite thorough analysis, no reported SNPs were identified in *A. lumbricoides* and *N. americanus* samples. These findings suggest a potential lack of diversity in the β-tubulin gene within the examined populations.

**Conclusions:** The findings suggest that the parasite species circulating in the South Indian region are susceptible to benzimidazoles. To the best of our knowledge, this is the first attempt in India to evaluate the SNPs in two of the STH parasites using cost-effective conventional PCR and Sanger sequencing. Although biannual deworming has been implemented in India since 2015, no resistance to benzimidazoles has been observed. This method is suitable even in resource-limited settings. However, due to the limited sample size, future research with a larger sample size is essential to confirm these results and provide a more comprehensive understanding of the susceptibility of these parasites to benzimidazoles in the broader population.

**KEYWORDS:** β-tubulin gene; Benzimidazole; Anti-helminthic resistance

**Significance**

Benzimidazole resistance in helminths is linked to single nucleotide polymorphisms (SNPs) at codons 167, 198, and 200, which have been extensively documented in livestock. However, evidence of these mutations in humans is still scarce. To address this gap, we investigated the presence of these SNPs in helminths found in South India. Our study did not reveal any of the reported SNPs in the β-tubulin gene in samples of *Ascaris lumbricoides* and *Necator americanus*. These findings offer valuable insights that can support ongoing efforts to eliminate soil-transmitted helminth infections.

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## 1. Introduction

Soil-transmitted helminths (STHs) are gastrointestinal nematodes primarily prevalent in tropical and subtropical regions, particularly where sanitation and hygiene conditions are poor[1]. STH infections encompass *Ascaris (A.) lumbricoides*, *Necator (N.) americanus, Trichiuris trichiura*, and hookworms, affecting over 1.5 billion individuals worldwide, including 654 million school-age children and approximately 138 million antenatal women. These infections result in significant morbidity, especially among children who are particularly vulnerable[2].

To meet the WHO's 2030 targets for eliminating STH infections as a public health concern, many countries have adopted biannual mass deworming strategies[3]. Benzimidazoles, such as albendazole and mebendazole, are commonly prescribed anthelmintic drugs for treating STH infections. These drugs exert their effects by binding to the β-tubulin gene of helminth microtubules and inhibiting microtubule polymerization. Albendazole inhibits the glucose uptake and transport, thus reducing the glycogen stores of the parasite. Eventually this results in paralysis and death of the parasite[4].

Resistance to Benzimidazole group of anti-helminthic had been increasingly reported in livestock. The practice of deworming in livestock is on the rise in India, and repeated exposure to the drugs has led to the development of resistance in parasites[5]. Observation of mass deworming campaign countrywide have resulted in reduction in infection intensity in humans. However, the continued biannual mass deworming with a single drug may also contribute to the development of drug resistance to Benzimidazoles in parasite populations. Benzimidazole resistance occurs due to single nucleotide polymorphism (SNP) of codons 167, 198 and 200 in the β-tubulin isotype I gene. SNPs in the β-tubulin gene at codons 167 (TTC, TTT → TAC, TAT; phenylalanine to tyrosine), 198 (GAG, GAA → GCG, GCA; glutamic acid to alanine), and 200 (TTC → TAC; phenylalanine to tyrosine) have been associated with benzimidazole resistance in various helminths. Mutations associated with these SNPs in parasite have been suspected when there was lower efficacy for albendazole in individuals[6,7].

Now, cure rate and faecal egg count reduction are considered as a proxy measure for measuring efficacy of albendazole and of drug resistance. These methods are insensitive and can detect the resistance only when the alleles are at high levels[8]. Resistance in STH species among humans have been documented in various locations, including Southern Mozambique, Brazil, and Ghana. A study in Southern Mozambique showed that the SNPs in β-tubulin gene was detected in 23.0% of *Trichiuris trichiura* and 21.0% of *N.americanus* samples. In Brazil, no mutations were found at the codons 167 and 198 for *A. lumbricoides* but codons 198 and 200 of *N. americanus* showed the SNPs at 1.4% and 1.1% respectively[9-11].Therefore, it is crucial to ascertain whether parasites circulating in our region have also developed resistance to anti-helminthic drugs. If this resistance is confirmed, it becomes imperative to address this threat in order to achieve the objectives of STH elimination set for the year 2030. With this background our objective was to identify the reported SNPs in *A.lumbricoides* and N.americanus species with the conventional PCR and sequencing technique.

## 2. Materials and methods

### *2.1. Sample collection and screening for N.americanus and A.lumbricoides*

A total of 650 stool samples were collected from pregnant women and screened for the presence of *A. lumbricoides* and *N. americanus*. Identification of these STH species was done with the conventional PCR and these samples were utilised for the purpose of SNP identification. Of the 650 samples, 17 (2.6%) were positive for the genus *Ascaris* and 41 (6.3%) were positive for *Necator*. The detailed methodology of DNA extraction, primer designing and PCR screening for STH species identification was followed[12]. Samples positive for *A. lumbricoides* and *N. americanus* were further utilised for the purpose of identification of SNP at codon 167, 198 and 200 of the β-tubulin gene.

### *2.2. Primer designing for the identification of SNP*

We have designed and standardized two PCRs for *N. americanus*, one for the identification of SNP targeting codon 167 and second one targeting codons 198 and 200 regions of β-tubulin gene. A single PCR was standardized for *A. lumbricoides* to detect the SNPs at all three codon positions. The primers were designed using published DNA sequences of β-tubulin gene retrieved from NCBI (GenBank ID: EU182348.1[13] for *N. americanus* SNP in 167 , GenBank ID: AF453524.1[14] for SNP at 198 and 200 position of *N. americanus* and GenBank ID; FJ501301.1 for *A. lumbricoides*[15]. This allowed the identification of the variable common regions that were suitable for primer design. The primers were designed using NCBI primer 3 tool. The primers were designed 250 base pairs upstream and downstream of the site where the SNP had been reported. Conventional PCR was performed using Sure cycler 8800 (Agilent technologies) and Sanger sequencing was performed to obtain the sequences.

The PCR amplification was performed using Taq DNA Polymerase

**Table 1.** Primers, product size and PCR conditions to amplify the codon region of 167, 198 and 200 in *Necator americanus* and *Ascaris lumbricoides*.

| Parasites | Codon | Size | DNA sequence | Conditions | Reaction mix of 25 µL |
|---|---|---|---|---|---|
| *Necator americanus* | 167 | 518 | F: 5'- TCCGCCCTGATAACTACGTG -3'<br>R: 5'- CTCAGCGCCATTTCCCATAG -3' | Initial denaturation : 95℃ for 4 min<br>No. of cycles: 35<br>Denaturation: 95℃ for 40 s<br>Annealing: 60℃ for 45 s<br>Extension: 72℃ for 45 s | Master mix: 12.5 µL<br>Forward primer: 0.5 µL<br>Reverse primer: 0.5 µL<br>Nuclease free water: 6.5 µL<br>Target DNA: 5 µL |
| *Necator americanus* | 198, 200 | 588 | F: 5'- GTCGACTGTTGCGTTACGAG -3'<br>R: 5'- GGAACCATGTTGACAGCCAA -3' | Denaturation: 95℃ for 40 s<br>Annealing: 62℃ for 30 s | Master mix: 12.5 µL<br>Forward primer: 1.5 µL<br>Reverse primer: 1.5 µL<br>Nuclease free water: 4.5 µL<br>Target DNA: 5 µL |
| *Ascaris lumbricoides* | 167, 198, 200 | 586 | F: 5'- GGTGGAGGCACAGGATCTGGC-3'<br>R: 5'- CGTATGTGGGATTTGTAAGCT -3' | Annealing: 63℃ for 45 s | Similar to Necator codon 198 and 200 PCR |

master mix (2×) (A180301, Amplicon, Denmark) in a final volume of 25 µL. Initial denaturation, denaturation and number of cycles were consistent across all PCRs. However, variations observed in the annealing temperature and reaction mix. The following conditions were used for different PCRs (For *N. americanus* PCR for codon 167: Annealing 60 ℃ for 45 s, 0.5 µL of each forward and reverse primers; *N. americanus* PCR for codons198 and 200: Annealing 62 ℃ for 30 s and 1.5 µL of each forward and reverse primers; *A. lumbricoides* PCR: Annealing of 63 ℃ for 45 s and 1.5 µL of each forward and reverse primers). In all reactions, master mix of 12.5 µL, target DNA of 5 µL and DNA/RNAse free water was used to achieve the final volume of 25 µL. DNAs which were positive for *A.lumbricoides* and *N.americanus* was used as a positive control and DNA/RNAse free water was used as a negative control during the assay standardization. Samples which were negative for the target also used to check the specificity of the assay. Further information on the primer sequences used, thermal cycling conditions and product size are given in Table 1. PCR optimization of SNPs is shown in Figure 1.

### 2.3. PCR product visualisation

The PCR products was run on a 1.5% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer with ethidium bromide at 100 volts for 45 min and the images were captured in with a Gel Documentation system (BioRad®). Band sizes corresponding to the amplification were observed in the agarose gel. The PCR products were outsourced for sequencing (Eurofins Scientific) and the products were purified and subjected to Sanger sequencing.

### 2.4. Identification of SNP

The amplified products from conventional PCR targeting respective codons of the β-tubulin gene was sequenced. Forward and reverse sequences of each sample retrieved from sequencing were aligned using DNA Baser Assembler and final Contig sequence was generated[16]. These contig sequences were matched with the reference sequence obtained from NCBI using BioEdit software with the function of ClustalW multiple alignment and then checked for any mutations or SNPs[17]. EU182348.1 and AF453524.1 was used as a reference sequence for SNP analysis of codons 167 and 198, 200 of *N. americanus*. FJ501301.1 was used as a reference for *A. lumbricoides* β-tubulin gene.

### 2.5. Ethical approval

The study protocol was reviewed and approved by scientific advisory committee and Institute ethics committee (for human studies) of Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India. A written informed consent has been obtained from each participant before enrolment. Ethical approval has been taken with the number JIP/ IEC/ 2019/ 137, dated July 2, 2019.

## 3. Results

We have used 22 samples for the identification of SNPs at codon 167 and 15 samples for the identification of SNPs at codons 198 and 200 of β-tubulin gene of *N. americanus*. Three samples were used for SNP analysis of *A. lumbricoides*. The amplified regions of codons 167, 198 and 200 SNPs of *N. americanus* and *A. lumbricoides* were sequenced and aligned with MEGA software and no where the change in nucleotide sequence was observed. Reference sequence from NCBI was used to confirm the SNP analysis. Of all the obtained β-tubulin sequences, none of them were identified with the SNPs suggestive of nucleotide change at codons 167, 198 and 200.

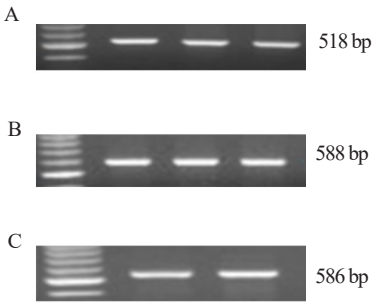



**Figure 1.** PCR optimization of identification of SNPs at codon 167, 198 and 200 of *Necator americanus* and *Ascaris lumbricoides*. A: PCR to amplify the SNPs at codon 167 of *N. americanus*; B: PCR to identify the SNPs at codon 198 and 200 of *N. americanus*; C: PCR to identify the SNP at codon 167, 198 and 200 of *A. lumbricoides*; Lane 1: 100 base pair ladder; Lane 2, 3 and 4: Positive sample.

## 4. Discussion

The present study assessed the SNPs associated with benzimidazole resistance at codons 167, 198, and 200 of *N. americanus* and *A. lumbricoides*. No previously reported SNP in the β-tubulin gene was detected in either parasite. Consistent with the results of Roose *et al.*, Diawara *et al.*, and Furtado *et al.*, who also reported the absence of mutations[6,18,19]. However, Furtado *et al.* used an ARMS-PCR method and reported a 0.5% mutation rate at codon 200 of *A. lumbricoides*[20]. Additionally, Zuccherato *et al.* employed PCR-RFLP and found mutations at codons 198 (1.4%) and 200 (1.1%) in *N. americanus*[9].

Notably, in southern part of India, resistance to albendazole has been documented in livestock, particularly in sheep and goats, as indicated by previous studies[21,22]. These investigations employed the Faecal Egg Count Reduction Test as a means of identifying albendazole resistance and also identified mutations in *Haemonchus* spp. Given that biannual deworming has been implemented in India since 2015 and has been ongoing for the past eight years, there exists a potential risk for the development of resistance. Therefore, it is important to maintain vigilant monitoring and surveillance of this situation. The role of animals and the environment is significant in the transmission of these parasites. Therefore, it is essential to keep this condition under surveillance to assess the prevalence of STH infections and to evaluate the impact of benzimidazole treatment to ensure its effectiveness. Furthermore, unsatisfactory human drug responses can be confirmed through SNP and mutation analysis.

Continued preventive measures reduced the parasite load and prevalence. Therefore, highly sensitive diagnostic techniques are crucial for diagnosis towards elimination. Also, achieving and sustaining the elimination of STH-related morbidity in preschool and school-age children by 2030 is feasible when benzimidazole resistance development is monitored.

To the best of our knowledge, this was the first attempt in India to evaluate the SNPs in two of the STH parasites using cost-effective conventional PCR and Sanger sequencing. It is suitable even in resource-limited settings. However, due to budget constraints, we could perform the SNP analysis for limited number of samples. Therefore, caution is warranted when generalising and interpreting these findings given the limited number of samples. Further large scale studies in different subgroups are the need of an hour to confirm the findings.

## 5. Conclusions

No mutations or SNPs associated with amino acid changes were detected in the β-tubulin gene of STH identified in the South Indian population. These findings suggests that parasites circulating in tat region are still susceptible to benzimidazoles. However, given the potential for emerging resistance, further large-scale, multi-regional studies with enhanced molecular screening techniques are essential to confirm these results and monitor the genetic evolution of STH populations.

## Conflict of interest statement

The authors claim there is no conflict of interest.

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## Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

## Authors' contributions

Ulaganeethi R, Rajkumari N, Chinnakali P, Saya GK were involved in concept and design of the study. Ulaganeethi R had done the data acquisition, analysis and interpretation of data and drafted the first version of the paper. Jasmine P helped in data acquisition and interpretation of results. Rajkumari N, Chinnakali P, Dorairajan G and Saya GK reviewed the paper critically and all the authors gave their consent to the version submitted for publication.

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