



Molecular detection and B-tubulin gene sequencing of *Enterobius vermicularis* and its relationship with resistance to albendazole in

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Abstract

Background: Enterobiasis, caused by *Enterobius vermicularis*, remains one of the most common helminthic infections among children worldwide. Albendazole is widely used for treatment; however, concerns regarding emerging resistance associated with mutations in the beta-tubulin gene have increased. Molecular diagnostic techniques provide improved sensitivity for parasite detection and resistance monitoring.

Objectives: This study aimed to detect *E. vermicularis* using molecular methods, analyze beta-tubulin gene polymorphisms, and investigate their possible relationship with resistance to albendazole.

Methods: A cross-sectional study was conducted on 100 stool samples collected from children aged 2–12 years. DNA was extracted using a stool DNA extraction kit, and PCR amplification targeted the 5S DNA gene and beta-tubulin gene. Positive beta-tubulin PCR products were subjected to Sanger sequencing and phylogenetic analysis. Statistical analysis was performed using SPSS, with significance considered at $p < 0.05$.

Results: PCR analysis identified *E. vermicularis* DNA in 30% of samples. The highest infection rate was observed among children aged 6–9 years (41.7%), showing a significant association with infection status ($p = 0.034$). Infection was also significantly associated with sampling location, particularly among schoolchildren ($p = 0.040$). Participants previously treated with albendazole demonstrated higher PCR positivity (39.1%) compared to untreated individuals (22.2%), although this difference was not statistically significant ($p = 0.105$). No significant association was found between ABO/Rh blood groups and infection risk ($p = 0.341$).

Conclusion: *E. vermicularis* was found to be significantly prevalent among the children, especially in school aged children through molecular identification. The beta-tubulin gene is a good molecular target to monitor the possible selection of resistance and to design future control measures.

Keywords: *Enterobius vermicularis*, albendazole resistance, beta-tubulin gene, PCR, molecular detection, children, enterobiasis.

Introduction

Enterobiasis is an extremely common nematode infection, caused by *Enterobius vermicularis*, which continues to be a public health problem, especially in children, around the world. Although the disease may seem inconsequential, the incidence and the ease of transmission within a home or institution make for the need to make effective therapeutic intervention (Chai et al., 2021). Albendazole and mebendazole are benzimidazoles that are the main medications used to treat the parasites. These agents work by binding to the beta-tubulin protein which prevents the polymerization of the microtubules, hence inhibiting vital cellular activities including parasite uptake of glucose and ultimately causing parasite death (Chai et al., 2021; Chulanetra & Chaicumpa, 2021).

Although these treatments have historically proved to be effective, there is increasing evidence of concern about development of resistance to these anthelmintics. Specific single nucleotide polymorphisms (SNPs) in the beta-tubulin gene (Prichard, 2007) have been associated with resistance in a number of human and veterinary nematodes. These genetic changes may cause decreased drug binding and clinical treatment failure (Chai et al., 2021; Kaneva et al ; 2026). Monitoring of development of resistance is important for the long-term sustainability of existing control measures (Chai et al., 2021; Prichard, 2007), and resistance has been reported in veterinary medicine but not in humans infected with *E. vermicularis*.

The advent of molecular diagnostic techniques has revolutionized the detection and characterization of parasitic infections. The molecular techniques (e.g., nested PCR and Sanger sequencing) can precisely identify and conduct phylogenetic analysis of *E. vermicularis* genotypes, which is not possible with traditional microscopic tools, as this latter technique may be less sensitive (Kaneva et al ; 2026). Sequencing of the beta-tubulin gene can be used to identify potential molecular markers linked to drug resistance, which will serve as a baseline to monitor the transmission dynamics and therapeutic efficacy (Prichard, 2007; Kaneva et al ; 2026). The objectives of this study are to assess the genetic diversity of *E. vermicularis* using molecular detection and gene sequencing techniques and to explore the relationship between molecular detection of *E. vermicularis* and the resistance to albendazole.

Materials and Methods

2.1 Study Design and Ethical Considerations

The present study aimed at identifying *Enterobius vermicularis* and the analysis of mutations of the beta-tubulin gene. All participants or their legal guardians gave informed consent before the samples were collected. Patient information was kept anonymous and patient confidentiality was ensured for all data.

2.2 Materials, Reagents, and Equipment

Specialized laboratory equipment was used in the study such as a T100 Thermal cycler (Bio-Rad, USA), Nanodrop spectrophotometer (Thermo Scientific, UK) and high-speed cold centrifuge (Eppendorf, Germany). The chemical reagents used were GoTaq Green PCR Master Mix (Promega, USA) and the Presto™ Stool DNA Extraction Kit (Geneaid, Taiwan). Tables 3-1 and 3-2 list the equipment and kits.

2.3 Sample Collection and Clinical Assessment

Stool samples were obtained from those who were suspected of infection. Patients were classified according to their clinical response to treatment to investigate the relationship with the albendazole resistance. Samples were brought to the lab in cooling boxes and kept at -20°C until they could be used for DNA extraction.

2.4 Molecular Detection of *Enterobius vermicularis*

3.4.1 Genomic DNA Extraction

DNA was extracted from fecal samples using the Presto™ Stool DNA Extraction Kit. The procedure involved:

1. Sample (100µl) was placed in bead-beating tubes with 800µl of ST1 buffer, heated to 70°C for 5 minutes and then vortexed for 10 minutes.
2. After centrifugation 500 µl of supernatant was pipetted into 800 µl of ST3 buffer and run through a GD column.
3. Washing and Elution: Wash Buffer was poured into the column and the column was centrifuged at 16000 x g. Elution Buffer (100 µl) preheated was used for the elution of purified DNA.

2.4.2 DNA Quality Control

The quality and quantity of the isolated DNA was determined by a Nanodrop spectrophotometer. The absorbance ratios of 260/280nm were taken and nuclease-free water was used as a blank to confirm that the DNA was fit for PCR amplification.

2.5 Polymerase Chain Reaction (PCR)

2.5.1 Primer Design

Specific primers were utilized for the detection of the 5s rDNA gene (340 bp) and the beta-tubulin gene (450 bp).

- **5s rDNA:** F: 5'-TAGACAGTGCAAGGCTGTGC-3' / R: 5'-TGCTTAGGTGCTGTTGCTGA-3'
- **beta-tubulin:** F: 5'-TAACTCACTCGCTGGGTGGA-3' / R: 5'-TTGTTGACCGATACGCAGCT-3'

3.5.2 Amplification Protocol

PCR reactions (25 µl) were prepared containing 12.5 µl Master Mix, 2 µl of each primer, and 3 µl of DNA template. The thermal cycling conditions included:

- Initial denaturation: 95°C for 5 min.
- 35 cycles: 95°C for 30s, 58°C for 30s, and 72°C for 2 min.
- Final extension: 72°C for 5 min.

2.6 Electrophoresis and Visualization

PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. Samples were run in 1X TBE buffer at 100 V and 80 mA for 1 hour. A 100 bp DNA ladder was used to confirm product sizes, and bands were visualized using a UV Transilluminator.

2.7 beta-tubulin Gene Sequencing and Bioinformatics

To identify mutations related to albendazole resistance, positive beta-tubulin PCR products were sent to Macrogen (Korea) for Sanger sequencing.

1. **Sequence Analysis:** Genetic variations were identified using **MEGA version 6.0**.
2. **Phylogenetic Analysis:** Sequences were aligned via ClustalW, and evolutionary distances were calculated using the UPGMA method with Maximum Composite Likelihood.

3. **GenBank Submission:** Homology was checked via NCBI BLAST, and confirmed local isolates were submitted to the NCBI-Genbank database for accession numbers.

2.8 Statistical Analysis

Data were analyzed using SPSS software. The correlation between beta-tubulin gene polymorphisms and clinical resistance to albendazole was evaluated using Chi-square test. A P-value of < 0.05 was considered statistically significant.

Results

Table 1: Demographical data of the study population (N = 100)

Variable	Category	Number	Percentage
Sex	Male	50	50.0%
	Female	50	50.0%
Residence	Urban	56	56.0%
	Rural	44	44.0%
Location	School	62	62.0%
	House	34	34.0%
	KG	4	4.0%
Age Groups	2-5 years	38	38.0%
	6-9 years	48	48.0%
	10-12 years	14	14.0%
	Mean $\pm$ SD	6.62 $\pm$ 2.48	

The ratio of male to female participants was perfectly balanced in Table 1 with 50% of male and 50% of female. More of the subjects lived in urban (56%) than in rural areas (44%). The sampling area was divided between schools (62%), private houses (34%) and kindergartens (4%). Participants' ages varied from 2 to 12 years, the mean age was 6.62 ± 2.48 years, and the most represented age group was 6–9 years (nearly half of the population, 48%).

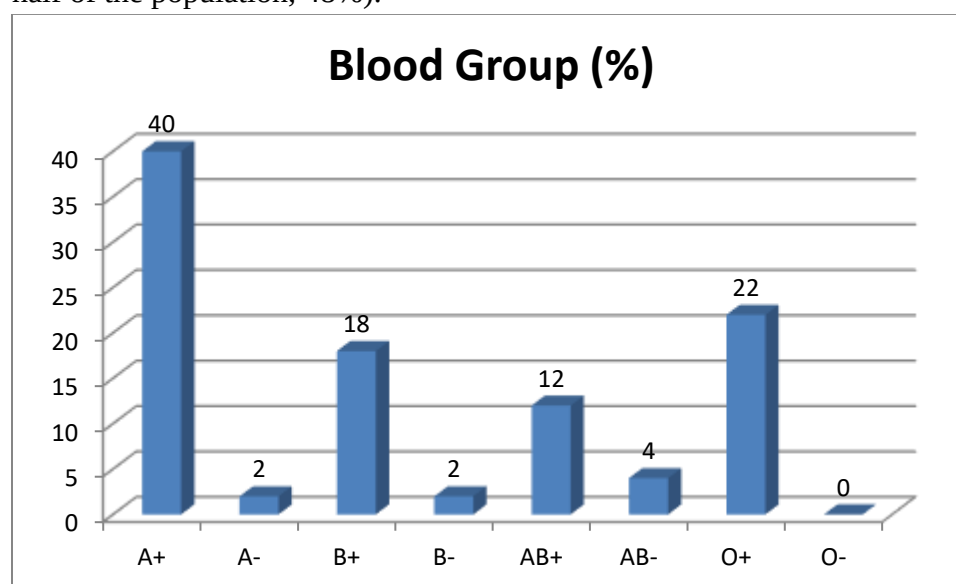


Figure 1: Distribution of Blood Groups among the study population

As seen in figure 1, most of the study population had Rh positive blood type (92%) and the most common group was Rh A+ (40%) followed by Rh O+ (22%) and Rh B+ (18%). Compared to this, Rh-negative blood groups were significantly less seen, accounting for only 8% of the total sample, with AB- being the most common blood group among the negative blood groups (4%) and no participant was found to be O- in this study.

Table 2: Distribution of the study population according to previous Albendazole treatment (N = 100)

Previous Albendazole Treatment	Number	Percentage
Yes	46	46.0%
No	54	54.0%
Total	100	100.0%

As shown in Table 2, almost half (46%) of the study participants had previously used albendazole, and 54% had never used the drug. The availability of this distribution allows a comparative study of the molecular detection and beta tubulin gene sequencing of treated and untreated individuals, which aims to find potential beta-tubulin gene markers linked to *Enterobius* resistance to the anthelmintics used.

Table 3: PCR results for the detection of *Enterobius vermicularis* (N = 100)

PCR Result	Number	Percentage
Positive	30	30.0%
Negative	70	70.0%
Total	100	100.0%

According to Table 3 (molecular analysis), 30 of the 100 samples (30%) were positive and 70 of them (70%) were negative for *Enterobius vermicularis* DNA..

Table 4: Association between demographic data and PCR results for *E. vermicularis* (N = 100)

Variable	Category	Positive n(%)	Negative n(%)	Total	p-value
Sex	Female	14 (28.0%)	36 (72.0%)	50	0.827
	Male	16 (32.0%)	34 (68.0%)	50	
Age Group	2-5 years	6 (15.8%)	32 (84.2%)	38	0.034*
	6-9 years	20 (41.7%)	28 (58.3%)	48	
	10-12 years	4 (28.6%)	10 (71.4%)	14	
Residence	Rural	16 (36.4%)	28 (63.6%)	44	0.312
	Urban	14 (25.0%)	42 (75.0%)	56	
Location	House	6 (17.6%)	28 (82.4%)	34	0.040*
	KG	0 (0.0%)	4 (100.0%)	4	
	School	24 (38.7%)	38 (61.3%)	62	

* Significant at $p < 0.05$

Demographic factors were analysed and showed significant association with PCR positivity for both age groups and sampling locations ($p < 0.05$). Specifically, children in the age group of 6-9 years had the highest infection rate (41.7%) and those of age 2-5 had the lowest (15.8%). Likewise, the sampling location was significantly correlated with infection, with the highest infection rate in schools (38.7%) followed by houses (17.6%) and kindergartens (0%). On the contrary, no statistical difference was found

between the infection rates of males and females ($p = 0.827$) and urban and rural residents ($p = 0.312$), indicating that gender and residential area (urban vs. rural) do not directly affect the risk but age and communal environment (school) do.

Table 5: Association between blood groups and PCR results for *E. vermicularis* (N = 100)

Blood Group	Positive n	Negative n	Total	p-value
A+	14 (35.0%)	26 (65.0%)	40	0.341
A-	0 (0.0%)	2 (100.0%)	2	
B+	4 (22.2%)	14 (77.8%)	18	
B-	0 (0.0%)	2 (100.0%)	2	
AB+	6 (50.0%)	6 (50.0%)	12	
AB-	0 (0.0%)	4 (100.0%)	4	
O+	6 (27.3%)	16 (72.7%)	22	
Total	30 (30.0%)	70 (70.0%)	100	

The study of correlation of blood group with presence of *Enterobius vermicularis* revealed highest positivity in AB+ blood group (50.0%) followed by A+ blood group (35.0%). Interestingly, the parasite was not detected in all participants who had a blood group of Rh-ve (A-, B-, AB-). These differences in distribution, however, were not significant as the statistical test gave a p value of 0.341 suggesting no significant association between the blood group type and the risk of infection with *E. vermicularis*. This indicates that blood group antigens could not be taken as a determinant in the susceptibility of children to this parasite infection in the sample analysed.

Table 6: Association between PCR results and previous Albendazole treatment (N = 100)

Previous Albendazole Treatment	Positive n(%)	Negative n(%)	Total	p-value
Yes	18 (39.1%)	28 (60.9%)	46	0.105
No	12 (22.2%)	42 (77.8%)	54	
Total	30 (30.0%)	70 (70.0%)	100	

The analysis reveals that the participants who had ever used albendazole had a higher rate of PCR positivity (39.1%) than those who had not ever used albendazole (22.2%). Although this difference indicates that previously treated subjects have a greater risk of remaining infected (or reinfected) with the parasite, the statistical analysis did not reach the 0.05 threshold (p -value = 0.105). This means that the association is not statistically significant at the 0.05 level of the sample. Thus, in this study group, there was a high proportion of previously treated cases who were also positive, which suggests that the history of treatment was not sufficient to determine the current state of infection in this instance and warrants further molecular study to confirm treatment resistance at the genetic level, through beta-tubulin gene sequencing..

Discussion

A prevalence of 30% for *Enterobius vermicularis* was seen in the present study. It is similar to the prevalence of enterobiasis being one of the most prevalent helminth diseases in young children worldwide, regardless of geographic location (Rossi et al;2024;Chulanetra & Chaicumpa.2021). Our results showed that the age group 6-9 years had the highest infected rate (41.7%) ($p = 0.034$), consistent with recent large-scale multi-center studies that suggested school environments could contribute to what has been called “within-school clustering” effects, meaning transmission takes place in a tight “pocket” and not randomly throughout the community (Zhang et al ; 2024; Chai et al ; 2021). Persistent prevalence in pediatric cohorts (Zhang et al ; 2024; Chai et al ; 2021) despite some reported decline in prevalence over the past 5 years due to social factors (schooling, high contact environments).

Therefore, one of the goals of this study was to explore the association between Albendazole treatment history and current infection status as a clinical marker of possible drug resistance. The data revealed that 39.1 % of the individuals taking albendazole previously were positive with PCR tests while 22.2 % of those who had not taken albendazole were positive with PCR tests. This is a higher rate in the previously treated patients, suggesting treatment failure, but it was not statistically significant ($p = 0.105$). This reflects some current concerns in contemporary parasitology that high levels of drug pressure, such as those imposed through mass deworming programmes, are likely to be associated with the selection of resistant strains, albeit that in high prevalence areas clinical treatment failure may be complicated by re-infection (Vercruysse et al;2022;Cringoli et al.,2021).

Considering the beta-tubulin gene as the main target of benzimidazoles (BZs), the molecular characterization was carried out. Single nucleotide polymorphisms (SNPs) at codons F167Y, E198A, and F200Y are the main sites of BZ resistance in different nematode species (Vercruysse et al;2022;Roose et al;2021) of humans and animals. In the clinical setting, microscopy is still the usual method, although it is far less sensitive to detect these genetic markers or even to detect low level infections, as is the molecular approach employed in this study (Avramova et al;2023). The fact that several members of the beta-tubulin gene family exist in the parasite also makes monitoring resistance difficult because genes may express differently at different stages of the parasite's life cycle (Roose et al;2021). Therefore, by using Sanger sequencing and UPGMA phylogenetic analysis, we have a more comprehensive basis to identify genetic markers of resistance that could be present early in the resistance process, even if not obvious from clinical observations (Vercruysse et al;2022;Roose et al;2021).

The last factor studied was host related, which was the ABO and Rh blood groups. Although the positivity rate of AB+ group was relatively higher (50.0%), the association was not statistically significant ($p = 0.341$) indicating that blood group antigens do not have significant influence on individual susceptibility to *E. vermicularis* (Chai et al;2021). Rather, poor hand hygiene and household overcrowding are the most significant predictors of infection (Chai et al;2021). The results highlight the importance of molecular surveillance of beta-tubulin mutations in future deworming programmes, but also highlight the need to focus on breaking transmission cycles in schools and the home environment, immediately (Zhang et al;2024;Avramova et al ; 2023).

Conclusion

The present study revealed a significant prevalence rate of 30% among children, most vulnerable in which was found to be the age group of 6-9 years ($p=0.034$). Patients with a previous history of albendazole use had higher infection positivity (39.1%) as compared to untreated patients (22.2%), but the difference was found to be statistically insignificant ($p=0.105$). This indicates possible high reinfections rates or resistance. Molecular analysis showed that the beta-tubulin gene is a suitable target for monitoring resistance, and there was no significant effect of biological factors, such as blood groups, on infection risk.

Recommendations

1. To have a uniform beta tubulin sequencing protocol to identify early genetic markers of drug resistance (SNPs).
2. Focus public health efforts on handwashing and sanitation within primary schools (6–9 year olds).
3. Don't treat each family member separately to avoid re-infection ("ping-pong effect").
4. Give a second dose of albendazole, which is mandatory to kill the larvae which have hatched after 14 days.
5. Carry out larger longitudinal studies to more explicitly correlate specific genetic mutations with clinical treatment failure.

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