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Single nucleotide polymorphisms in the *β-tubulin gene* family of *Ascaris lumbricoides* and their potential role in benzimidazole resistance: a systematic review

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Introduction: The most common soil-transmitted helminthic infection is caused by *Ascaris lumbricoides (A. lumbricoides).* Approximately 4 billion people are at risk of infection globally. The World Health Organisation recommends the administration of benzimidazole- containing deworming drugs (Albendazole and Mebendazole) to all susceptible populations. Due to this high drug pressure, these parasites may develop resistance to current benzimidazole drugs. The *β-tubulin gene* family is the target gene for benzimidazole deworming drugs. This systematic review aimed to highlight work that explored the genetic mutations in the *β-tubulin gene* family of *A. lumbricoides* that are associated with potential benzimidazole resistance.

Methods: An electronic search of several online databases was used to extract eligible articles using specific keywords related to the topic of interest.

Results: The majority of ascariasis infections occur in the subtropical and tropical regions of sub-Saharan Africa, the Americas and East Asia, although not enough studies were done to extensively cover this geographical range. In the *β-tubulin gene* family of *A. lumbricoides* the mutations at codons F200Y (TTC/Phenylalanine to TAC/Tyrosine), E198A (GAG, GAA/Glutamic acid to GCG, GCA/Alanine) and F167Y (TTC, TTT/Phenylalanine to TAC, TAT/Tyrosine) were associated with potential benzimidazole resistance.

Discussion: Resistant mutations were found in *A. lumbricoides* samples at codon F167Y from Haiti, Kenya and Panama. The first evidence of the mutation at codon F200Y was observed in Brazil. The codon E198A mutation was the least prevalent and most undetected.

Conclusion: There is a serious shortage of studies investigating the prevalence of *β-tubulin gene* family mutations in *A. lumbricoides* populations from endemic areas; this is a serious concern as resistance will negatively impact current mass drug administration programmes.

KEYWORDS

***Ascaris lumbricoides*, *β-tubulin gene* polymorphisms, benzimidazole drug resistance, Mebendazole and Albendazole, treatment efficacy**

Introduction

The World Health Organisation (WHO) has advocated that all countries move towards the elimination of neglected tropical diseases (NTDs), including soil-transmitted helminthiasis, which afflicts more than 1.5 billion people globally (24% of the global population) (1). The most prevalent soil-transmitted helminths (STHs) currently affecting the world's population are *Necator americanus, Strongyloides stercoralis, Trichuris trichiura, Ancylostoma duodenale*, and *Ascaris lumbricoides* (2, 3). Ascariasis is the most common STH infection caused by *A. lumbricoides*, an intestinal roundworm, and infects an estimated 1.2 billion people worldwide (4) with a global approximation of 4 billion people at risk of infection (5). The heavy burden of *A. lumbricoides* infections causes more than 60,000 deaths annually (6) and an estimated 1.8 - 10.5 million disability-adjusted life years (DALYs) (7).

The majority of ascariasis infections occur in the subtropical and tropical areas of sub-Saharan Africa, the Americas and East Asia, particularly poverty-stricken areas with poor access to clean water and poor sanitary conditions (4). The prevalence of *A. lumbricoides* infections in sub-Saharan Africa is 13.6%, 15.6% in South America, and 18% in Southeast Asia and South Asia (8–10). Comparatively, with the introduction of modern waste management systems and sanitation in the United States (US) at the start of the 20th century, ascariasis prevalence has significantly decreased (11). However, cases of *A. lumbricoides* infections still occur as a result of emigration to the US and travel from the US to foreign countries where the prevalence of ascariasis infection is high (11). According to Hong et al. (12), additional factors that worsen the situation in developing countries include natural disasters, social instability, poor hygiene practices, inadequate healthcare facilities and systems, civil wars, and low-quality healthcare (12).

The current prevention strategy put forward by the WHO is the mass deworming drug administration to all susceptible populations, particularly school children, who experience the highest rates of parasite infections (13). In large-scale deworming programs, benzimidazole drugs such as Albendazole (a single dose of 400 mg taken orally) or Mebendazole (a single dose of 500 mg taken orally or two doses daily of 100 mg taken orally over three days) remain the current mainstays of treatment (7). These initiatives aim to reduce the morbidity caused by intestinal worms (7). In 2012, the London Declaration, led by the WHO Director General and the Bill Gates Foundation, committed funding and effort for the elimination of 10 NTDs, including STHs, by 2030 (14). The landmark feature of this declaration is that the world's 13 leading pharmaceutical companies pledged to donate free anthelminthic drugs to all endemic countries. This exponentially accelerated the uptake of the mass drug administration (MDA) in many endemic countries. By 2021, 62 countries reported on the implementation of large-scale MDA treatment programmes for at least one of the targeted diseases. By that year, overall, 429 million were treated for soil-transmitted helminthiases (14). The only drugs used for the MDA in all the recipient endemic countries are the benzimidazoles (Mebendazole and Albendazole).

Continuous administration of the same drug treatment in single doses to large numbers of people provides opportunities for the evolution of potential drug resistance (15). Due to this high drug pressure among communities, *A. lumbricoides* may develop resistance to current benzimidazole drugs. The target for these benzimidazole drugs in *A. lumbricoides* is the *β-tubulin* proteins, where resistance is likely to develop, highlighting the need for surveillance systems to identify genetic mutations associated with benzimidazole resistance (16). However, there is a lack of knowledge on the mutations in *β- tubulin gene* family of *A. lumbricoides* that could potentially confer benzimidazole resistance and prevents the development of such tools (16).

Thus, this systematic review aims to gather information from published research literature globally regarding the prevalence of single nucleotide polymorphisms (SNPs) in the *β-tubulin gene* family of *A. lumbricoides* namely codons F167Y (TTC, TTT/Phenylalanine to TAC, TAT/Tyrosine), F200Y (TTC/Phenylalanine to TAC/Tyrosine) and E198A (GAG, GAA/Glutamic acid to GCG, GCA/Alanine) that may confer resistance to benzimidazole, methods used for SNP detection and also discuss the effect of Mebendazole and Albendazole drug resistance and treatment efficacy in endemic populations.

Methods

This systematic review collected relevant data from previous literature regarding SNPs found in the *β-tubulin gene* family of *A. lumbricoides* at codons F167Y, F200Y and E198A that could

potentially confer benzimidazole resistance to current treatment regimens of Albendazole and Mebendazole. A narrative approach was followed to review relevant and available data on this topic.

Literature search strategy

ScienceDirect, Google Scholar, MEDLINE, PubMed and Institute for Scientific Information (ISI) Web of Knowledge databases were searched using the following keywords: '*Ascaris lumbricoides*', '*A. lumbricoides*', '*β-tubulin gene* mutations', 'benzimidazole resistance', 'F200Y', 'E198A', 'F167Y' and 'single nucleotide polymorphisms'. Individual keywords and a combination of the keywords were used to search for relevant literature. The relevant data was analyzed and reported following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (17) guidelines. This study included all English language-published literature globally from January 1990 to December 2022.

Study selection, quality of studies and data extraction

Appropriate literature was first selected by their titles, abstracts and full-text according to the exclusion and inclusion criteria by the main author (T.R.) and eligibility of the literature to be included in this study was approved and checked for discrepancies and duplicates by the two co-authors (P.N. and Z.L.M.K.). The quality of the relevant data extracted from the literature and presented in this study was separated into four categories: high quality, moderate quality, low quality and very low quality (18). This was assessed using the grading of recommendations, assessment, development and evaluations (GRADE) (19).

Inclusion criteria

- Literature reporting on SNPs in the *β-tubulin gene* family of *A. lumbricoides* and the prevalence of these polymorphisms at codons F200Y, E198A and F167Y.
- Literature reporting on benzimidazole resistance in the *β-tubulin gene* family of *A. lumbricoides* due to SNPs at codons F200Y, E198A and F167Y.
- Literature published in all countries and in English from January 1990 to December 2022.
- Case-controlled studies, cross-sectional studies and cohort-appropriate studies.
- Published data in five databases.

Exclusion criteria

- Literature published prior to January 1990.
- Articles not published in English.
- Reviews, Comments and editorials.
- Literature that is not relevant to *A. lumbricoides* and *β-tubulin gene* SNPs at codons F200Y, E198A and F167Y.

A total of 282 articles were found using the search criteria in the above-mentioned databases. Of the 282 articles, 192 were retained after the removal of duplicates. Further evaluation of the articles based on the eligibility criteria narrowed down the article selection to 12 articles. Finally, 8 of the 12 articles were selected based on *A. lumbricoides* SNPs (F200Y, E198A and F167Y) *β-tubulin gene* family data and benzimidazole resistance. A systematic flow diagram for the selection of articles chosen for this systematic review based on initial identification, screening, eligibility and final selection is represented in Figure 1.

Results

Information regarding studies that were included, their relevant study population and *A. lumbricoides* sample type used for genetic analysis is described in Table 1. Table 2 presents the main results of the articles that were reviewed, assessed and included in this systematic review.

Discussion

This is the first systematic review that presents data on specific SNPs in codons F200Y, E198A, and F167Y in the *β-tubulin gene* family of *A. lumbroicoides* globally. Benzimidazole resistance in the in the *β-tubulin gene* family of helminths has been associated with amino acid substitutions from phenylalanine to tyrosine (TTC/Phenylalanine to TAC/Tyrosine) at codon 200 (F200Y SNP) (28). A similar SNP at codon 167, which also causes an amino acid substitution of phenylalanine to tyrosine (TTC, TTT/Phenylalanine to TAC, TAT/Tyrosine) (F167Y SNP) was also observed (29). Although rare, occasionally, a SNP at codon 198 can cause an amino acid substitution of glutamic acid to alanine (GAG, GAA/Glutamic acid to GCG, GCA/Alanine) (E198A SNP), which is also associated with benzimidazole resistance (26, 30).

Furtado et al. (26) screened the *β-tubulin* isotype 1 gene for SNPs at codon F200Y in *A. lumbricoides* samples obtained from seven states in Brazil; they observed a mutation at codon F200Y at 0.5% frequency (n=4/854) (26). Despite the low frequency observed, this is the first observation of an SNP detected at codon F200Y in *A. lumbricoides*, and the presence of this SNP is indicative of the potential of these parasite populations to develop resistance to current treatments and possibly at higher levels in the near future (26). This study did not indicate whether the sample population investigated had been previously exposed to benzimidazole treatment.

Roose et al. (27) observed at least seven different *β-tubulin genes* in *A. lumbricoides* samples from Ethiopia, Tanzania and Belgium, as well as an eighth putative *β-tubulin* encoding gene (27). The *β-tubulin* isotype 1 gene and the *β-tubulin* isotype 2 gene were shown to be highly expressed during the entire parasite life cycle and, therefore,

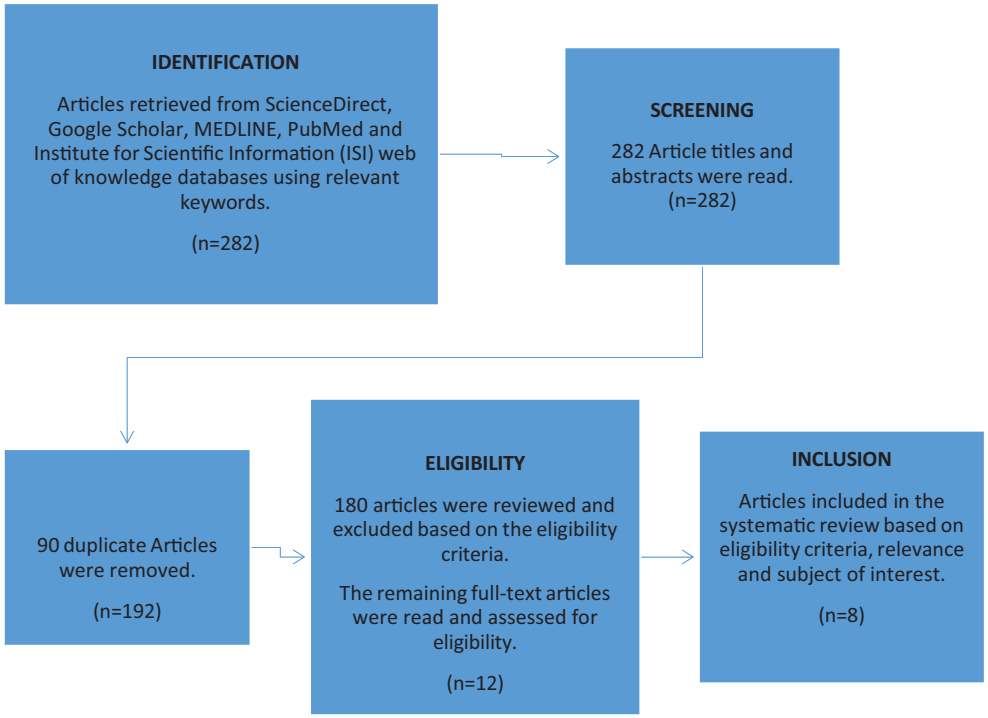

FIGURE 1
Systematic flow diagram representing the article selection process used to include relevant data from the initial identification, screening, eligibility and final selection of articles based on A. lumbricoides SNPs (F200Y, E198A and F167Y) β-tubulin gene family data and Benzimidazole resistance.

are more likely to play an important part in benzimidazole resistance (27). This β-tubulin phylogeny is fairly complex; hence, it is challenging to establish a common nomenclature across species for the *β-tubulin genes* and makes the comparative analysis for the role of these genes in benzimidazole resistance difficult (27). Presently, in human *A. lumbricoides* species, there is a lack of knowledge about the genes that are associated with potential benzimidazole resistance in the *β-tubulin gene* family.

Four *β-tubulin gene* isotypes of *A. lumbricoides* were investigated by Krücken and colleagues (23) in Rwandan school children. They reported a reduced efficacy to benzimidazole treatment. Their observed cure rate was 69.9% (95% CI 63.4-75.4%) by Mini-FLOTAC test and 88.6% (95% CI 83.8 - 92.2%) by wet mount microscopy and the 95% calculated confidence intervals for the fecal egg count reductions (FECRs) were less than 95% indicating a reduced efficacy towards treatment. These results observed cause for the suspicion of benzimidazole resistance; however, no SNPs at codons F167Y, E198A and F200Y were observed in the four investigated *β-tubulin gene* isotypes (23).

Rashwan and colleagues (22) developed a SmartAmp2 method that targeted polymorphisms in the *β-tubulin* isotype 1 gene of *A. lumbricoides*. They detected the SNP TTC>TAC in codon F167Y in Haiti; however, no information was given with regard to whether infected populations were previously exposed to benzimidazole (22). Consequently, for *A. lumbricoides*, the existing data is not yet well-defined for the use of these SNPs as a marker for benzimidazole resistance (31). In addition to this, due to the poor understanding of the roles of the different isotopes in the *β-tubulin gene* family that contribute to benzimidazole resistance in the *Ascaris* species, it is challenging to interpret and determine whether the absence of the possible resistance-conferring SNPs is relevant in a particular *β-tubulin gene* isotype without prior knowledge about other possibly relevant β-tubulin loci (27).

The characterization of the genes and potential mutations in the *β-tubulin* family of *A. lumbricoides* species will provide a framework for future research to determine the possible role of the *β-tubulin gene* SNPs in conferring benzimidazole resistance and its prevalence in a more methodical manner than previously possible (12). Diwara et al. (21) observed an SNP at codon F167Y at high frequencies from Kenya, Panama and Haiti populations that received benzimidazole treatment (21). In pre-treatment samples from Haiti, the homozygous resistance-type (HRT) (TAC/TAC at a 40% frequency) and heterozygous type (TTC/TAC at a 60% frequency) were observed. All post-treatment samples were found to be heterozygotes (TTC/TAC at a 100% frequency), and the ERR from the Kato-Katz technique was 99.9% (95% CI 99.5–100.0)[21]. In Kenya, the genotype frequency of codon F167Y observed was predominantly the HRT (TAC/TAC at a frequency of 72.7%) in pre-treatment samples. Post-treatment samples showed no significant difference to the HRT (TAC/TAC at a frequency of 72.7%) observed. However, there was a significant increase of heterozygotes (TTC/TAC, from 4.5% to 21.1% ($p<0.001$)) and a decrease of the homozygous susceptible-type (HST) (TTC/TTC, from 22.7% to 5.3% ($p<0.001$). The ERR calculated from the Kato-

TABLE 1 Summary of articles included in this systematic review investigating the prevalence of the F200Y, E198A and F167Y SNPs in the *β-tubulin gene* family of *A. lumbricoides*, the target sample population of each study and their respective sample of choice from which genomic DNA was extracted.

Author	Year	Title	Sample Population	*A. lumbricoides* sample type
Diawara et al. (20)	2009	*"Assays to Detect β-Tubulin Codon 200 Polymorphism in Trichuris trichiura and Ascaris lumbricoides"*	Samples collected from school-aged children in Kenya, Panama, Zanzibar and Uganda	*A. lumbricoides* genomic DNA extracted from adult worms and eggs isolated from adult worms or human fecal samples (n=158)
Diawara et al. (21)	2013	*"Association Between Response to Albendazole Treatment and β-tubulin Genotype Frequencies in Soil-transmitted Helminths"*	Samples collected from children and adults in Haiti, Schoolchildren in Kenya and Preschool children in Panama	Genomic DNA extracted from *A. lumbricoides* eggs in human fecal samples pre- (n=221) and post (n=104) Albendazole treatment
Rashwan, Scott and Prichard (22).	2017	*"Rapid Genotyping of β-tubulin Polymorphisms in Trichuris trichiura and Ascaris lumbricoides"*	Samples collected from Haiti and Panama	Genomic DNA extracted from *A. lumbricoides* eggs in human fecal samples. (n=74)
Krücken et al. (23)	2017	*"Reduced efficacy of Albendazole against Ascaris lumbricoides in Rwandan schoolchildren"*	Samples collected from Rwandan schoolchildren aged 6 - 10 years	Genomic DNA extracted from *A. lumbricoides* eggs (n=144) in human fecal samples
Zuccherato et al. (24)	2018	*"PCR-RFLP screening of polymorphisms associated with Benzimidazole resistance in Necator americanus and Ascaris lumbricoides from different geographical regions in Brazil"*	Samples collected from six Brazilian states	Genomic DNA extracted from *A. lumbricoides* eggs (n=601) from human fecal samples
Matamoros et al. (25)	2019	*"High Endemicity of Soil-Transmitted Helminths in a Population Frequently Exposed to Albendazole but No Evidence of Antiparasitic Resistance"*	Samples collected from children aged 0.6 – 13years in Honduras	*A. lumbricoides* genomic DNA extracted from adult worms in human fecal samples (n= 8)
Furtado et al. (26)	2019	*"First identification of the Benzimidazole resistance-associated F200Y SNP in the beta- tubulin gene in Ascaris lumbricoides"*	Samples collected from seven Brazilian states	Genomic DNA extracted from *A. lumbricoides* eggs (n=845) in human fecal samples
Roose *et al* (27).	2021	*Characterization of the β-tubulin gene family in Ascaris lumbricoides and Ascaris suum and its implication for the molecular detection of Benzimidazole resistance*	Schoolchildren that were between 7 to 14 years of age, from Ethiopia and Tanzania Belgium.	*A. lumbricoides* genomic DNA extracted from adult worms (n=106) from human stools.

Katz was 97.3% (95% CI, 0.0–100.0%) and McMaster technique was 80.3% (95% CI, 0.0–100.0%)[21]. Pre-treatment samples from Panama predominantly had the HRT (TAC/TAC at a frequency of 97.7%), and the genotype with the lowest frequency was the HST (TTC/TTC at a frequency of 2.3%). In post-treatment samples, there were no significant changes in the genotype percentages observed, with a frequency of 96.7% and 3.2%, respectively. The ERR estimated from the FLOTAC was 89.8% (95% CI, 75.8–97.3%) (21).

Therefore, the HRT allele (TAC) at codon F167Y was at a high frequency pre – and post- treatment and remained in the population after the infection was cleared in Kenya and Panama. However, in samples from Haiti, the HRT genotype TAC/TAC frequency significantly increased after treatment (21). According to the WHO

TABLE 2 Summary of articles included, identifying the prevalence of F200Y, E198A and F167Y polymorphisms in the *β-tubulin gene* family of *A. lumbricoides*.

Aim of study for *A. lumbricoides* samples	Molecular methods for *A. lumbricoides* samples	SNP investigated in *A. lumbricoides*	Main outcome	Ref.
To characterize the *β-tubulin* genomic sequences around codon F200Y in *A. lumbricoides*, and develop pyrosequencing assays for the detection of phenylalanine 200 or tyrosine 200 in the *β-tubulin* gene of *A. lumbricoides*.	- Genomic DNA extracted from adult worms, eggs from adult worms and eggs from fecal samples - Initial isolation of the *β-tubulin* gene of *A. lumbricoides*: A nested PCR was done to amplify the cDNA of *A. lumbricoides* with two degenerate sets of primers that were designed by using the conserved regions of the *β-tubulin* gene of six related nematodes (*Necator americanus, Haemonchus contortus, Trichuris trichiura, Brugia malayi, Circumcincta, Teladorsagia and Onchocerca volvulus*). - The outer sense primer and the outer antisense primer were used to amplify the cDNA. The PCR product was then used as a template for the next amplification using the sense and antisense nested primers. - The PCR products were then cloned into the pCR2.1 TOPO vector. - To isolate the 3' end of *β-tubulin* cDNA, two gene-specific primers were designed in a nested PCR reaction with oligo adaptor primers. - The resulting fragments were purified and ligated into pGEM-T cloning vector and then sequenced from both directions with SP6 and T7 vector primers. - Phylogenetic analysis was performed to identify the relationship between *β-tubulin* sequences of 13 nematodes. - Pyrosequencing DNA assay optimization: control plasmids based on the amplification of a small portion of the *β-tubulin* genomic sequence from *A. lumbricoides* were constructed - Pyrosequencing was performed for the detection of SNPs in the genomic DNA extracted from *A. lumbricoides* samples, and the resulting genotype sequences were confirmed by conventional sequencing.	Codon F200Y (TTC/Phenylalanine to TAC/Tyrosine)	No detection of SNP at codon F200Y Genotypes T/T (100%) were observed from *A. lumbricoides* samples from Zanzibar and Uganda Genotypes T (100%) were only observed from *A. lumbricoides* egg pool samples from Panama	(20)
To determine the drug efficacy of Albendazole against *A. lumbricoides* and to examine the frequency of SNPs that are associated with Albendazole resistance at each time point by conducting examinations on stools and genotyping the *β-tubulin* gene of *A. lumbricoides* eggs collected pre-and post-treatment in Haiti, Kenya, and Panama.	- Haiti: Stool samples were collected and analyzed before treatment with Albendazole (n = 353); stool samples were then collected and analyzed two weeks after Albendazole treatment (400 mg Albendazole and 6 mg/kg Diethylcarbamazine (DEC)) (n= 317). Stool analysis and egg isolation were done by using a modified McMaster technique. - Kenya: Stool samples were collected and analyzed before treatment with Albendazole (n = 128); Stool samples were then collected and analyzed seven days after Albendazole treatment (400 mg Albendazole) (n= 92). Stool analysis and egg isolation were done by using a modified McMaster technique and Kato-Katz technique. - Panama: Children received two treatments of Albendazole 9 months apart (children between one - two years received 200 mg of Albendazole; children between three - five years received 400 mg of Albendazole) Stool samples were collected and analyzed before receiving the second treatment (n = 270) and thereafter collected and analyzed three weeks after the second treatment (n= 222). Stool analysis and egg isolation were done using the FLOTAC and Kato-Katz techniques. - Egg reduction rates (ERR): calculated as the ratio of the difference between the mean of the pre-treatment and post-treatment fecal egg count (FEC) to the pre-treatment mean, expressed as a percentage, with its respective confidence interval (CI) - *A. lumbricoides* genomic DNA was extracted from all eggs isolated. - PCR was performed on genomic DNA to amplify the *β-tubulin* gene SNPs (E198A, F200Y	Codon F200Y (TTC/Phenylalanine to TAC/Tyrosine), E198A (GAG, GAA/Glutamic acid to GCG, GCA/Alanine) and F167Y (TTC, TTT/Phenylalanine to TAC, TAT/Tyrosine)	Detection of SNP F167Y (TTC, TTT/Phenylalanine to TAC, TAT/Tyrosine) Haiti: At codon F167Y, pre-treatment samples were homozygous resistance-type (HRT) (40%) and heterozygous (60%); post-treatment samples were only observed to be heterozygotes (100%). ERRs from the Kato-Katz technique were 99.9% (95% CI 99.5–100.0) Kenya: Codon F167Y, the genotype frequency observed was predominantly the HRT (72.7%). Post-treatment samples showed no significant difference to the (72.7%) HRT observed. However, there was a statistically significant increase of heterozygotes (from 4.5% to 21.1% ($p<0.001$)) and a decrease in the homozygous susceptible-type (HST) (from 22.7% to 5.3% ($p<0.001$)). The ERR calculated from the Kato-Katz technique was 97.3% (95% CI, 0.0–100.0%), and the	(21)

(Continued)

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TABLE 2 Continued

Aim of study for *A. lumbricoides* samples	Molecular methods for *A. lumbricoides* samples	SNP investigated in *A. lumbricoides*	Main outcome	Ref.
	and F167Y) -Genotyping was done with the Pyrosequencer, and primers were designed to amplify single egg DNA using the PyroMark Assay Design Software. -SNPs investigated were validated with real-time PCR and conventional Sanger sequencing.		McMaster technique was 80.3% (95% CI, 0.0–100.0%). Panama: At codon F167Y, the HRT was observed (97.7%). The genotype with the lowest frequency was the HST (2.3%). In post-treatment samples, there were no significant changes in the genotype percentages observed (96.7% and 3.2%, respectively). The ERR estimated from the FLOTAC was 89.8% (95% CI, 75.8–97.3%)	
To develop new genotyping assays to detect the presence of *β-tubulin* SNPs (F200Y, E198A and F167Y) in *A. lumbricoides* samples from Haiti and Panama.	- *A. lumbricoides* genomic DNA was extracted from eggs in fecal samples. - Mutant-type (MT) and Wild-type (WT) plasmids were engineered for each parasite species SNPs (F200Y, E198A and F167Y) and used as DNA templates for assay optimization. - PCR and Sanger sequencing were used to verify the presence of WT alleles at codons F200Y, E198A and F167Y. - Site-directed mutagenesis was used to engineer MT plasmids carrying mutations at codons F200Y, E198A and F167Y. - SmartAmp2 assay: primer sets were created specifically for the amplification and detection of the E198A, F167Y and F200Y SNPs of the *β-tubulin* isotype 1 gene. - SmartAmp2 assay development and optimization were carried out to identify the best primer sets for the WT and MT genotypes -Sanger sequencing and pyrosequencing were used to confirm genotyping results from the SmartAmp2 assay for *A. lumbricoides* SNPs (F200Y, E198A and F167Y)	Codon F200Y (TTC/Phenylalanine to TAC/Tyrosine), E198A (GAG, GAA/Glutamic acid to GCG, GCA/Alanine) and F167Y (TTC, TTT/Phenylalanine to TAC, TAT/Tyrosine)	Detection of SNP F167Y (TTC, TTT/Phenylalanine to TAC, TAT/Tyrosine) for *A. lumbricoides* in Haiti, frequency unknown -Development of SmartAmp2 method that targets polymorphisms in the *β-tubulin* gene of *A. lumbricoides*	(22)
To evaluate the treatment efficacy of Albendazole on *A. lumbricoides* infections and determine the occurrence of benzimidazole resistant β-tubulin SNPs in four isotypes of *A. lumbricoides* among Rwandan school children.	- Stool samples were collected pre-treatment (a single dose of 400 mg Albendazole) alongside routine school-based deworming programmes and post-treatment (7-10 days after treatment). - *A. lumbricoides* infected stool samples were analyzed by mini-FLOTAC and direct wet mount microscopic examination, eggs were isolated, and genomic DNA was extracted. - PCR amplification was performed using primers designed for codons F167Y, E198A and F200Y. - PCR amplicons were analyzed through agarose gel electrophoresis, and the resulting PCR products were purified. - Sequencing was performed using primers to characterize the sequences at the different isotypes and codons of the *β-tubulin* genes. - Chromatograms for sequences were visualized using BioEdit software and sequences were manually analyzed for the presence of SNPs E198A, F200Y and F167Y in the *β-tubulin* gene of *A. lumbricoides* as well as for missense SNPs within the amplicons. - Fecal egg count reduction (FECR), Cure rate (CR) and their respective CIs were calculated as the proportion of children who tested positive for *A. lumbricoides* infection before treatment survey, which became negative 7- 10 days after treatment.	Codon F200Y (TTC/Phenylalanine to TAC/Tyrosine), E198A (GAG, GAA/Glutamic acid to GCG, GCA/Alanine) and F167Y (TTC, TTT/Phenylalanine to TAC, TAT/Tyrosine)	No detection of SNP's at codon F200Y, F167Y and E198A At codons F167Y and F200Y: TTC genotypes were observed encoding phenylalanine. At codon E198A: differences were observed between isotypes. Isotypes 1 and 4 were (GAG), and isotypes 1.2 and 2 were (GAA). Since both genotypes were found to encode for glutamate, there is no evidence that SNPs confer benzimidazole resistance. - CR was 69.9% (95% CI 63.4- 75.4%) by Mini-FLOTAC and 88.6% (95% CI 83.8 - 92.2%) by wet mount microscopy. - FECR was 75.4%. the 95% calculated CIs for the FECR were 55.4-88.8% by bootstrapping, 50.4-87.8% using sample variance and 75.0-75.7% by applying a Markov Chain Monte Carlo Bayesian approach.	(23)

(Continued)

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TABLE 2 Continued

Aim of study for *A. lumbricoides* samples	Molecular methods for *A. lumbricoides* samples	SNP investigated in *A. lumbricoides*	Main outcome	Ref.
To investigate the presence of the molecular markers that are associated with resistance to benzimidazole in *A. lumbricoides* samples obtained from six different states in Brazil.	- Eggs of *A. lumbricoides* were isolated from human stool samples, and DNA was extracted. - Primer3 software was used to create primers based on the *A. lumbricoides β-tubulin* nucleotide sequences from WormBase ParaSite (*A. lumbricoides*: PRJEB4950, Assembly GCA_000951055.1) and GenBank (EU814697.1) - Controls for the presence (MT) and absence (WT) of the SNP for each codon were constructed. - *In silico* analysis was done on *β-tubulin* nucleotide sequences of *A. lumbricoides* retrieved from WormBase ParaSite and GenBank databases. - NEBcutter V2.0 tool was used to find restriction sites in *A. lumbricoides* to differentiate between the MT and WT alleles in codons F167Y and E198A. - Using one primer pair, an initial PCR was performed to amplify the codons F167Y and E198A. Thereafter, another primer pair was used to perform a nested PCR using the initial PCR product as a template. - PCR-RFLP was performed using the enzymes *RsaI* and *BmsI* to distinguish between the unmutated and mutated codons F167Y and E198A of *A. lumbricoides*. - PCR-RFLP positive samples with mutations were sequenced for validation purposes. All amplification runs included a negative control sample.	Codon E198A (GAG, GAA/ Glutamic acid to GCG, GCA/Alanine) and F167Y (TTC, TTT/ Phenylalanine to TAC, TAT/Tyrosine)	No detection of SNP's F167Y and E198A	(24)
To determine the frequency of benzimidazole-resistant SNPs (F200Y, F167Y and E198A) in the *β-tubulin* gene *A. lumbricoides* worms from children frequently exposed to Albendazole in Honduras.	- Stool samples were collected from children (n=106) and examined using the Kato-Katz technique. According to the WHO recommendations *A. lumbricoides* infections are regarded as light: 1–4999 Eggs per gram (epg); Moderate: 5000–49,999 epg; and heavy >50,000 epg. - Eight children (n=8) with heavy and moderate *A. lumbricoides* infections were treated over four consecutive days, and stool samples were collected from the children within 24 hours after each treatment. -Treatment for *A. lumbricoides* infections consisted of Piperazine (single dose of 75 mg/kg); Albendazole (single dose of 400mg); Albendazole (single dose of 400mg); Albendazole (single dose of 400mg) from days one – four respectively. Treatment for *A. lumbricoides* and *Trichuris trichiura* mixed infections consisted of Pyrantel-oxantel (single dose of 10 mg/kg) and Piperazine (single dose of 75 mg/kg); Pyrantel-oxantel (single dose of 10 mg/kg); Pyrantel-oxantel (single dose of 10 mg/kg); Albendazole (single dose of 400mg) from days one – four respectively. - *A. lumbricoides* genomic DNA was extracted from adult worms found in the stool samples. - SNP-specific primers targeting the *A. lumbricoides β-tubulin* gene were used to amplify segments around codons F200Y, F167Y and E198A. - A semi-nested PCR was performed to amplify the fragments around codons F200Y and E198A. While a single PCR was used to amplify the fragments around codon F167Y. - The same primers used in the PCR amplifications were used to sequence the PCR products, and sequence alignment was done using MUSCLE through the Geneious software, R8.1 version. - SNP (F200Y, E198A and F167Y) frequencies were reported as percentages and the Kato-Katz results observed were used to calculate the point prevalence of *A. lumbricoides* infections with 95% CI.	Codon F200Y (TTC/ Phenylalanine to TAC/Tyrosine), E198A (GAG, GAA/Glutamic acid to GCG, GCA/Alanine) and F167Y (TTC, TTT/ Phenylalanine to TAC, TAT/Tyrosine)	No detection of SNP's at codon F200Y, F167Y and E198A 40 genomic sequences of *A. lumbricoides* were generated and analyzed for the 3 SNPs; all codons were observed to be monomorphic Out of 106 stool samples examined, *A. lumbricoides* prevalence was 17.0% (CI: 95%, 9.83%, 24.13%)	(25)

(Continued)

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TABLE 2 Continued

Aim of study for *A. lumbricoides* samples	Molecular methods for *A. lumbricoides* samples	SNP investigated in *A. lumbricoides*	Main outcome	Ref.
To standardize a molecular tool based on an amplification refractory mutation system (ARMS-PCR) and screen for the F200Y SNP in the *β-tubulin* isotype 1 gene Of *A. lumbricoides* from samples obtained from seven different states in Brazil	- *A. lumbricoides* DNA was extracted from single eggs in stool samples. - Primer3 software was used to create primers based on the *A. lumbricoides β-tubulin* isotype 1 sequence available from the Genbank database (under the accession number FJ501301.1.) - Mutant-type (MT) and Wild-type (WT) plasmids for *A. lumbricoides* SNP F200Y were constructed and used as controls. - PCR amplification was performed, and amplified fragments were visualized under agarose gel electrophoresis to verify PCR products - Plasmid products were excised from the gel, purified, cloned and sequenced to verify the presence of the mutation - ARMS-PCR was used to analyze the *β-tubulin* isotype 1 codon F200Y of *A. lumbricoides*. - Sanger sequencing was performed for confirmation of samples with mutated alleles.	Codon F200Y (TTC/ Phenylalanine to TAC/Tyrosine)	Detection of SNP at codon F200Y at a frequency of 0.5% (n=4/854).	(26)
To comprehensively characterize gene mutations (F200Y, E198A and F167Y) associated with drug resistance, in the *β-tubulin* genes of *Ascaris lumbricoides* and *Ascaris suum* in Tanzania, Ethiopia and Belgium.	-A single dose of Albendazole (400mg) was administered orally to children over three consecutive days (Ethiopia) or for one day (Tanzania). - *A. lumbricoides* worms were collected consecutively from stools within 24 hours for seven days, and DNA was extracted. - Analyses of published genomes (from NCBI) of *A. lumbricoides and A. suum* were used to characterize and compare the *β-tubulin* gene families. - The transcription profiles of the various *β-tubulin* genes of *A. suum* throughout its life cycle were analyzed using RT-PCR on cDNA and RNA-sequencing - The intra- and inter-species genetic diversity, as well as the presence of benzimidazole resistance-associated SNPs (F200Y, E198A and F167Y) in *the β-tubulin* genes of *A. lumbricoides* and porcine benzimidazole-drug-exposed worms samples were assessed through deep amplicon sequencing.	Codon F200Y (TTC/ Phenylalanine to TAC/Tyrosine), E198A (GAG, GAA/Glutamic acid to GCG, GCA/Alanine) and F167Y (TTC, TTT/ Phenylalanine to TAC, TAT/Tyrosine)	No detection of SNP's at codon F200Y, F167Y and E198A Seven *β-tubulin* gene isotypes were observed in *A. lumbricoides* and an eighth putative *β-tubulin* encoding gene. -The *β-tubulin* isotype 1 gene and the *β-tubulin* isotype 2 gene were shown to be highly expressed during the entire parasite life cycle and, therefore, are more likely to play an important part in benzimidazole resistance	(27)

Ref, References; TT, HST (TTC/TTC); AA, HRT type (TAC/TAC), TA, heterozygous (TTC/TAC).

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standardized thresholds for Albendazole, the drug is highly effective if the ERRs are between 92% - 100%; the estimated ERRs in this study showed the treatments to be successful (21). This may suggest that the SNP at codon F167Y may not impact the efficacy of the drug, although more testing needs to be done to confirm this (21).

Matamoros et al. (25) investigated the prevalence of SNPs in the *β-tubulin gene* family of 40 genomic sequences from *A. lumbricoides* samples that had received prior treatment with Albendazole in Honduras, where STH infections are highly endemic, no mutations at codons F200Y, F167Y and E198A were detected, and all sequences were observed to be monomorphic. They calculated the prevalence of *A. lumbricoides* infections in their sample population to be 17.0% (CI: 95%, 9.83%, 24.13%) (25). Similarly, Zuccherato et al. (24) collected *A. lumbricoides* samples from six Brazilian states to determine the presence of SNPs at codons E198A and F167Y in the *β-tubulin gene* family of *A. lumbricoides*; they were also unsuccessful in detecting the presence of any mutations. The authors did not state if the target population was previously exposed to benzimidazole treatment (24). Diwara A. et al. (20) developed a pyrosequencing assay to detect for an SNP at codon F200Y in the *β-tubulin gene* family of *A. lumbricoides* samples from Zanzibar, Uganda and Panama. The authors did not mention whether the sample population received prior treatment with benzimidazole. No mutations were observed, all genotypes found in Uganda and Zanzibar were T/T at a frequency of 100%, and genotypes observed from Panama samples were only T at a 100% frequency (20). Although these studies were unsuccessful in detecting SNPs in *A. lumbricoides* samples, the molecular methods developed for SNP detection will prove to be valuable tools for future research.

Benzimidazole drugs bind to *β-tubulin* proteins and disrupt the microtubule function (23). Thus, mutations in the *β-tubulin gene* family can alter the amino acid sequence of the *β-tubulin* protein, making the *β-tubulin* protein less sensitive to benzimidazoles (32). Which in turn makes the parasite resistant to the drug. An *in silico* study by Jones et al. (33) showed that in the *β-tubulin gene* family of the *Ascaris* genus, E198 is a crucial amino acid for benzimidazole binding of *β-tubulins* and, the E198A and F200Y SNPs both confer resistance by disrupting this key anchor point however, they did not observe any effect of the F167Y mutation (33).

The *β-tubulin gene* families of many helminths, such as *Trichinella spiralis*, filarial nematodes and *H. contortus*, have been used to perform *in silico* docking studies (32, 34).These studies noted the protein conformational changes that take place when resistance mutations are present and their effects on drug interactions (32–34). Presently, little work has been done to apply these methods to *A. lumbricoides*, and few studies have done research into the changes that may possibly occur between the individual *β-tubulin* isotypes within a genus or species by detecting the frequency of the F200Y, F167Y and E198A SNPs (20–27). Additionally, in human *T. trichiura* correlation of these SNPs in the *β-tubulin gene* with poor response to benzimidazole treatment has been reported, Rashwan and colleagues (22) observed that the frequency of SNPs (E198A and F167Y) increased significantly in individuals who responded poorly to Albendazole treatment as opposed to individuals who responded well (20, 22).

Given the current MDA programmes implemented around the globe (13), and taking into account the evidence of the existence of these SNPs in the *β-tubulin gene* family of *A. lumbricoides* presented by the studies analyzed in this systematic review (21, 22, 26) as well as evidence of these same SNPs conferring benzimidazole resistance in the *β-tubulin genes* of other veterinary nematodes (20, 22, 30), the possibility of benzimidazole resistance developing in *A. lumbricoides* cannot be ruled out. Future studies should focus on screening for these SNPs at codons F167Y, E198A and F200Y in the *β-tubulin gene* family of *A. lumbricoides* where infection rates are high and MDA programmes are ongoing. Future research also needs to be done on the treatment efficacy of Albendazole and Mebendazole in relation to these specific SNPs (F167Y, E198A and F200Y) in the *β-tubulin gene* family of *A. lumbricoides*. The data generated from such studies will be crucial in gaining a deeper understanding of the status of benzimidazole resistance in *A. lumbricoides* and could possibly incorporate approaches for prevention, management and treatment, thereby decreasing the economic and health burden due to benzimidazole anthelmintic resistance around the globe.

Limitations

This narrative systematic review comprehensively analyzed published literature and included all nationalities, races, genders and geographical locations that complied with the search strategy and criteria used. However, there were a few limitations to this review. The literature included was limited to articles published in English; articles in other languages were excluded. Due to the lack of research on this topic, the literature included in this review does not have a broad geographical range or target population. The reported studies were only done in Africa: Kenya, Zanzibar, Uganda, Tanzania, Ethiopia and Rwanda; the Americas: Panama, Haiti, Brazil and Honduras. *A. lumbricoides* infection is also highly endemic in East Asia; however, no studies regarding this topic were found to be done in this population. Future studies that target highly endemic *A. lumbricoides* populations and larger sample populations may provide more insightful findings. Only a few articles included in this review were able to detect SNPs in *A. lumbricoides*, and some of the articles included in this review were not able to clearly define the SNP genotype frequencies and *A. lumbricoides* prevalence. Furthermore, literature analyzing the genomic DNA from pooled egg samples and adult *A. lumbricoides* worms did not differentiate the differences found between genotypes from eggs and worm samples. An analysis of whether SNPs are more easily detected from egg samples or worm samples would be beneficial for future studies. Only 50% (n=4/8) of the studies included had stated whether the populations investigated received prior exposure to benzimidazole treatment, and none of the articles included in this systematic review had indicated if the target populations had received multiple exposures

to benzimidazole treatment. This information is vital as single exposure to treatment may not necessarily exert significant pressure on the development of benzimidazole resistance-associated SNPs. Populations undergoing MDA programmes that have been exposed to benzimidazole treatment multiple times are ideal for researching the selection pressure placed on *A. lumbricoides* samples by treatment methods while also considering possible naturally occurring SNPs.

Conclusion

In conclusion, the reported results on the occurrence of SNPs (F200Y, E198A and F167Y) and their potential role in conferring benzimidazole resistance in the *β-tubulin gene* family of *A. lumbricoides* are still limited compared to the number of countries that have accepted the donated benzimidazole drugs for the mass treatment of children in endemic countries. More extensive research needs to be aimed at characterizing and detecting SNPs in codon 200 (F200Y: TTC/Phenylalanine to TAC/Tyrosine), codon 198 (E198A: GAG, GAA/Glutamic acid to GCG, GCA/Alanine) and codon 167 (F167Y: TTC, TTT/Phenylalanine to TAC, TAT/Tyrosine) in the *β-tubulin gene* family of *A. lumbricoides*. This research should be widely applied in endemic and peri-urban areas where *A. lumbricoides* infection is the most predominant. This would be of value in determining if there are genetic variations of *A. lumbricoides* circulating within specific populations and if *A. lumbricoides* is developing resistance to the current benzimidazole treatment methods. Although the majority of ascariasis infections occur in the subtropical and tropical areas of sub-Saharan Africa, the Americas and East Asia, not enough studies were done to extensively cover this geographical range. More research should be done in areas that are highly endemic for *A. lumbricoides* infections. Resistant mutations were found in *A. lumbricoides* samples at codon F167Y (TTC, TTT/Phenylalanine to TAC, TAT/Tyrosine) from Haiti, Kenya and Panama at high frequencies. The first evidence of the resistant SNP at codon F200Y (TTC/Phenylalanine to TAC/Tyrosine) was also observed in Brazil at a low frequency. This could negatively impact current MDA programmes and could potentially propagate further studies on alternative treatment regimes. Identifying and characterizing these SNPs and their potential role in benzimidazole resistance will also be a key link for future research in treatment methods for resistant genotypes of the *β-tubulin gene* family of *A. lumbricoides*, such as developing treatments that target different genes of the parasite or developing alternative treatment methods.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

TR: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. PN: Formal Analysis, Project administration, Supervision, Validation, Writing – review & editing. ZM: Formal Analysis, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

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